UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4712
www.usuhs.mil

APPROVAL SHEET

Title of Thesis: "Role of Regulatory Immune Responses in the Pathogenesis of Schistosomiasis"

Name of Candidate: Mazen Makarem
Molecular and Cell Biology Program
Master of Science
Date:

Thesis and Abstract Approved:

Charles Via, M.D.
Department of Pathology
Committee Member

7/15/08
Date

Stephen Davies, Ph.D.
Department of Microbiology & Immunology
Committee Member

7/15/08
Date

Tao-Yiao Wu, Ph.D.
Department of OB/GYN
Committee Member

7/15/08
Date

The author hereby certifies that the use of any copyrighted material in the thesis manuscript entitled:

"Role of Regulatory Immune Responses in the Pathogenesis of Schistosomiasis"

is appropriately acknowledged and, beyond brief excerpts, is with the permission of the copyright owner.

Mazen Makarem

Mazen Makarem
Molecular and Cell Biology Program
Uniformed Services University

Abstract

Title of Dissertation:

Role of Regulatory Immune Responses in the Pathogenesis of Schistosomiasis

Mazen N. Makarem

Thesis directed by:

Stephen J. Davies, B.V.Sc, Ph.D

Assistant Professor, Department of Microbiology and Immunology

Schistosomiasis leads to significant morbidity and mortality, associated primarily with the production of eggs by schistosomes. Our previous studies, using a murine model of *Schistosoma mansoni* infection, demonstrated that schistosome development in the mammalian host require the presence of host $CD4^+$ T cells to complete their development normally. Our goal was to elucidate whether the activation of $CD4^+$ T cells to schistosome worm antigens is necessary for the development of schistosomes in early infection. $CD4^+$ T cell responses that occur earlier in schistosome infection are of considerable interest because of their potential ability to kill migrating schistosomula and mediate protection against infection. We therefore initiated studies to better characterize the early $CD4^+$ T cell responses to schistosome worm antigens in greater detail. $CD4^+$ T cells from livers of 4 week infected mice expressed predominantly IL-10 mRNA and protein. IL-$10^{-/-}$ mice showed that IL-10 is responsible for regulating IFN-γ levels in early

infection. We also report that disruption of other regulatory mechanisms, such as Adenosine 2A receptor (A2AR) signaling, leads to the dysregulation of $CD4^+$ T cell responses to worm antigen, and that this dysregulation is mediated directly at the level of the $CD4^+$ T cells. Further, loss of A2AR expression exacerbated levels of schistosome egg-induced fibrosis. We also investigated the role of effector $CD4^+$ T cells on their ability to facilitate schistosome development. Priming by infection or vaccination does not interfere with the ability of transferred $CD4^+$ T cells to promote schistosome development in $RAG^{-/-}$ mice, suggesting that $CD4^+$ T cell responses are not critical to influencing parasite development. Moreover, in vaccinated mice, we provide evidence that $CD4^+$ T cells simultaneously mediate protection against schistosome infection and facilitate worm development. Finally, we show that inhibition of regulatory responses during early infection have some effect on parasite development. Together these results indicate that early schistosome infection induces a regulatory response in the host, possibly allowing *S. mansoni* worms to evade the immune response and establish a chronic infection. These findings suggest that overcoming regulatory responses in early infection could lead to development of more effective vaccines for preventing schistosome infection.

Hypothesis and Specific Aims

Our previous studies, using a murine model of *Schistosoma mansoni* infection, demonstrated that schistosomes specifically require the presence of host $CD4^+$ αβ T cells to complete their development normally. However, the mechanism by which $CD4^+$ T cells facilitate schistosome development is not known. Therefore, we sought to investigate whether the activation of $CD4^+$ T cells to schistosome worm antigens is necessary for the development of schistosomes. Because the primary function of $CD4^+$ T cells is to recognize and mount responses to foreign peptide antigens presented in the context of major histocompatibility complex (MHC) class II molecules, **we hypothesize that $CD4^+$ T cell responses to *S. mansoni* worm antigens during early infection facilitate the development of schistosome parasites**. To test this hypothesis, our specific aims are:

Specific Aim #1: To characterize the $CD4^+$ T cell response to *S. mansoni* worms in early infection. While the overall T cell response to egg antigens in the later phases of *S. mansoni* infection has been determined to be a strong Th2 response, the comparatively limited amount of published data available suggest the response to worm antigens during the early stages of primary infection is a relatively minor Th1 response. In this aim we will specifically assess the $CD4^+$ T cell response to worm antigens in early infection.

Specific Aim #2: To determine the effect of antigen-experienced $CD4^+$ T cells, as compared to naïve $CD4^+$ T cells, on growth and development of *S. mansoni* worms. This aim will be addressed by adoptively transferring $CD4^+$ T cells from infected donors into $RAG^{-/-}$ recipients, to assess their ability to restore the growth and development of *S. mansoni* worms.

Specific Aim #3: To determine if immune regulatory mechanisms in early infection allow *S.mansoni* worms to evade the immune response. Published data suggest that IL-10 interferes with vaccine-induced immunity to schistosomes. Further, our preliminary data show that IL-10 production by $CD4^+$ T cells is elevated early during *S. mansoni* infection, suggesting a possible role for regulatory T cell responses during worm development. To assess this possibility, the regulatory response in infected animals will be disrupted to determine its effect on *S. mansoni* worm establishment and development.

Chapter 1

Rapid Establishment of Regulatory T cell Responses by Schistosome Worms and the Non-Redundant Role of Adenosine Receptor 2A Signaling in Modulating $CD4^+$ T Cell Cytokine Production

(Manuscript in Preparation)

Mazen N. Makarem[1], Tuere Wilder[2], Lucia A. O. Fraga[1], Anna S. Tocheva[1], Erika W. Lamb[1], Bruce N. Cronstein[2] and Stephen J. Davies[1]

[1] Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814, USA

[2] Department of Medicine, New York University School of Medicine, 550 First Avenue, New York, NY 10016, USA

Abstract

Schistosome infection, which results in exposure of the host to a variety of different parasite life cycle stages, induces a diversity of $CD4^+$ T cell responses that change in character as the infection proceeds and the parasites mature. In contrast to schistosome eggs, which are potent inducers of Th2 responses, here we show that intravascular schistosome worms rapidly induce a systemic regulatory $CD4^+$ T cell response during the first four weeks of infection. In both liver and spleen, $CD4^+$ T cells from infected mice produced IL-10 in response to schistosome worm antigens, which served to modulate concomitant Th1 responses. Furthermore, we provide evidence for regulation of both Th1 and Th2 responses during schistosome infection by adenosine signaling through the A2A receptor. In the early stages of infection, A2AR signaling cooperated with IL-10 to limit IFN-γ production by $CD4^+$ T cells via a direct mechanism involving activation of A2ARs on $CD4^+$ T cells themselves. However, later in infection, A2AR signaling was found to be a negative regulator of egg-induced Th2 cytokine production and the subsequent Th2-mediated fibrosis associated with schistosome eggs. Together our data indicate that, during schistosome infection, multiple regulatory mechanisms are induced both by schistosome worms and eggs and that signaling by the "retaliatory metabolite" adenosine plays a protective role in limiting excessive fibrosis in response to schistosome eggs.

Introduction

Schistosomiasis, a neglected tropical disease caused by intravascular trematode parasites of the genus *Schistosoma*, afflicts over 200 million people worldwide, while a further 600 million individuals living in endemic areas are at risk of infection (Pearce and MacDonald, 2002). Schistosome infection leads to significant morbidity and mortality, associated primarily with the production of schistosome eggs, which embolize in the blood to various tissues and organs and cause chronic, granulomatous inflammation that disrupts tissue architecture and compromises organ function (Wynn et al., 2004). The pathology caused by egg deposition is mediated by $CD4^+$ T cell responses to egg antigens, which are strongly biased towards a Th2 phenotype (Pearce and MacDonald, 2002). Indeed, schistosome eggs and their antigens are potent autonomous inducers of Th2 responses (Vella and Pearce, 1992) that have been widely and successfully used as model Th2 antigens to investigate the induction of Th2 responses (Pearce, 2005). In schistosomiasis, egg-induced Th2 responses drive the accumulation of eosinophils (Sher et al., 1990b), alternatively activated macrophages (Herbert et al., 2004) and collagen (Wynn et al., 2004) in egg-associated granulomas. The Th2 cytokine interleukin (IL-) 13 plays a central role in the latter process (Wynn, 2003), which leads to one of the most severe consequences of schistosomiasis – extensive and largely irreversible fibrosis of essential organs such as the liver (de Jesus et al., 2004).

Because of their critical role in mediating the immunopathology associated with schistosomiasis (Wilson et al., 2007), egg-induced Th2 responses have deservedly received much attention and have contributed greatly to our understanding of Th2 cell development and function, in the context of helminth infections and in general. However,

$CD4^+$ T cell responses that occur earlier in schistosome infection, before the onset of egg deposition in the fifth or sixth week of infection, are also of considerable interest for two reasons. First, the developing juvenile schistosomes (schistosomula) are vulnerable to immunological attack and offer the best target for vaccines that aim to protect against schistosome infection (Hewitson et al., 2005). Indeed, the most successful experimental vaccine for schistosomiasis to date, consisting of skin exposure to radiation-attenuated infectious larvae (cercariae), targets migrating schistosomula during the first two weeks of subsequent challenge infection by inducing a protective Th1 response that eliminates schistosomula through a variety of effector mechanisms (James et al., 1998; Jankovic et al., 1999). Second, for reasons that remain unclear, $CD4^+$ T cells are required for the normal growth and development of *Schistosoma* species once they reach the portal vasculature, at least in mice (Davies et al., 2001). For these reasons, we initiated studies to characterize early $CD4^+$ T cell responses to schistosome worm antigens in greater detail, to better understand how $CD4^+$ T cells mediate protection against schistosome infection under some circumstances and yet facilitate the establishment of infection in others.

While the immune response induced by vaccination with irradiated cercariae has been well studied (Hewitson et al., 2005), there is considerably less data available on the response of naïve hosts to primary infection with non-irradiated cercariae. Previous studies found that splenocyte cultures from prepatently infected mice consistently produce IFN-γ in response to schistosome worm antigens (Pearce et al., 1991), leading to the widely accepted model that schistosome infection of naïve hosts begins with a relatively minor Th1 response to worm antigens that is then superseded by a vigorous

Th2 response to egg antigens around six weeks post infection (Pearce and MacDonald, 2002). Recently, a number of informative studies have expanded our understanding of interactions between normal cercariae and the immune system in the skin and indicate that cercarial invasion induces a transient, mixed Th1/Th2 response that is rapidly downregulated (Angeli et al., 2001; Hogg et al., 2003a; Hogg et al., 2003b). This rapid decline in Th responses may be due to exploitation of several host regulatory mechanisms by the parasites, in addition to release of a schistosome molecule with anti-inflammatory prostaglandin synthase activity (Angeli et al., 2001). Thus, there appear to be qualitative differences in the immune responses initiated at the site of infection in the skin and systemically, once the parasites enter the vasculature. Here we provide evidence that reconcile these disparate results by showing that the systemic type 1 $CD4^+$ T cell response to worm antigens is subject to considerable regulation by IL-10-producing $CD4^+$ T cells. These IL-10-producing $CD4^+$ T cells are induced by schistosome infection, do not express the regulatory T cell-specific transcription factor Foxp3, secrete IL-10 in response to schistosome worm antigens and co-localize anatomically with the developing schistosomes in the livers of infected animals. Further, we show that $CD4^+$ T cell responses to schistosome worms and eggs are subject to regulation by adenosine 2A receptor (A2AR) signaling, an innate regulatory mechanism active at sites of cellular injury and stress (Hasko and Cronstein, 2004). By comparing early responses to worms with later responses to eggs, we show that A2AR signaling modulates both Th1 and Th2 responses via A2ARs expressed on $CD4^+$ T cells and that disruption of A2AR signaling is associated with exacerbation of egg-induced pathology.

Materials and Methods

Mice

A2AR$^{-/-}$ and wild type controls on a 129B6 background were maintained in a breeding colony at the New York University animal facility. Six-wk-old male C57BL/6 IL-10$^{-/-}$ mice were purchased from Jackson. Wild type C57BL/6 mice were purchased from National Cancer Institute. All mice were maintained in a specific pathogen-free environment.

Schistosome infection and vaccination

Cercariae of *Schistosoma mansoni* (Puerto Rican strain) were obtained from infected *Biomphalaria glabrata* snails. For establishment of primary schistosome infection, mice were infected by immersion of the tail for 40 min in water containing 150 *S. mansoni* cercariae. For experiments where animals were vaccinated prior to infection, animals were first exposed three times at 5-7 week intervals to 500 *S. mansoni* cercariae irradiated with 50 krad from a ^{60}Co source. Mice were then infected as described and euthanized at 4 weeks post infection.

Parasite antigen Preparation

For preparation of soluble worm antigen (SWAP), adult *S. mansoni* worms (male and female) were suspended in ice-cold PBS and homogenized on ice. Insoluble material was removed by centrifugation at 13,200 x g for 45 min at 4°C, and the supernatant was filter-sterilized. Protein concentration was determined by the Bradford assay (BioRad,

Hercules, CA) on a Spectramax M2 micro plate spectrophotometer (Molecular Devices, Sunnyvale, CA) and frozen in aliquots stored –80°C. Soluble *S. mansoni* egg antigen (SEA) was provided by Dr. Jankovic (LPD, NIH).

CD4$^+$ T cell isolation

Single-cell suspensions of leukocytes were prepared from pooled spleens and pooled livers of infected mice by dissociating tissues through nylon cell strainers and lysing erythrocytes with ACK lysing buffer if necessary. For livers, 25 ml of 35% Percoll (GE Healthcare Bio-Sciences AB) was added to the single-cell suspension from each liver and centrifuged at 2000 rpm for 10 min at room temperature to separate the leukocyte pellet from the lower density hepatocytes. CD4$^+$ T cells were isolated by positive selection using magnetic anti-CD4 microbeads and MACS separation columns (Miltenyi Biotech, CA), following the instructions provided by the manufacturers. In some experiments, CD4$^+$ T cells were isolated by flow cytometry using a FACSAria cell sorter (BD Biosciences) so that other CD4$^+$ cells such as NKT cells and myeloid cells could be excluded using anti-NK1.1, anti-CD11c and anti-CD11b antibodies as previously described.

Gene expression analysis by real-time PCR

RNA was extracted from whole tissue or purified CD4$^+$ T cells using the RNeasy mini-kit (Qiagen) and RNA concentration was determined by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA (1 ug) was reverse-transcribed using the iScript Select cDNA Synthesis kit (Bio-Rad, Hercules, CA).

Real-time PCR was performed on a CHROMO 4 PTC-200 (Bio-Rad) using the iQ SYBR Green Supermix kit (Bio-Rad). Primer sequences used are as follows: IFN-γ: forward 5'-ATGAACGCTACACACTGCATC-3', reverse 5'-CCATCCTTTTGCCAGTTCCTC-3'; IL-4: forward 5'-GGTCTCAACCCCCAGCTAGT-3', reverse 5'-GCCGATGATCTCTCTCAAGTGAT-3'; IL-5: forward 5'-CTCTGTTGACAAGCAATGAGACG-3', reverse 5'-TCTTCAGTATGTCTAGCCCCTG-3'; IL-10: forward 5'-CTGGACAACATACTGCTAACCG-3', reverse 5'-GGGCATCACTTCTACCAGGTAA-3'; IL-12p40: forward 5'-AGACATGGAGTCATAGGCTCTG-3', reverse 5'-CCATTTTCCTTCTTGTGGAGCA-3'; IL-13: forward 5'-GCAACATCACACAAGACCAGA-3', reverse 5'-GTCAGGGAATCCAGGGCTAC-3'; A2AR: forward 5'-AGCCAGGGGTTACATCTGTG-3', reverse 5'-TACAGACAGCCTCGACATGTG-3'; 18s rRNA: forward 5'-CGGCTACCACATCCAAGGAA-3', reverse 5'-GCTGGAATTACCGCGGCT-3'. The comparative threshold cycle (C_T) of each gene of interest was then normalized to the sample's C_T for 18s ribosomal RNA using the Opticon Monitor 2 software and fold increase vs control samples calculated using the $\Delta\Delta C_T$ calculation.

In vitro restimulation of $CD4^+$ T cells

$CD11c^+$ splenic dendritic cells were isolated from non-infected mice using magnetic anti-CD11c microbeads and MACS separation columns. $CD4^+$ cells isolated as described above were then co-cultured with dendritic cells in T cell medium (TCM; RPMI 1640,

10% heat-inactivated fetal calf serum (FCS), 1000 U/ml penicillin, 10 μg/ml streptomycin, HEPES, 10mM; MEM Non-essential amino acids Solution, 10mM; L-glutamine, 200mM; Sodium pyruvate, 100mM; β-mercaptoethanol, 55mM) at a ratio of 10:1. Depending on experiment, SWAP or SEA was added to a final concentration of 50μg/ml or 20μg/ml respectively. Positive and negative control cultures received 1 μg/ml anti-CD3 antibody or no antigen, respectively. Following incubation for 72 h at 37 °C in 5 % carbon dioxide, supernatants were collected and immediately frozen at –80°C for subsequent determination of cytokine concentration.

Cytokine ELISAs

The concentrations of cytokines in culture supernatants were determined using sandwich ELISA kits from BD Biosciences/Pharmingen (San Diego, CA) and eBioscience following the instructions issued by the manufacturer. Briefly, for each cytokine, flat-bottomed plates (Immulon 2HB; Thermo, MA) were each coated overnight at 4 °C with the appropriate capture antibody diluted in carbonate-bicarbonate coating buffer (pH 9.5). Following an initial wash with PBS (pH 7.2) containing 0.05% Tween 20 (PBST; Sigma), the plates were blocked with 10% fetal calf serum (FCS) in PBS for 1 h at room temperature. Samples or standards were then added to the wells and the plates were incubated for 2 hours at RT. After incubation, the plates were washed and 100 μl of working detector containing biotinylated anti-mouse cytokine antibody and Streptavidin-horseradish peroxidase conjugate (HRP) was added in a one-step incubation for 1 hour. After 7-10 washes, the substrate solution TMB and hydrogen peroxide was added to each well and 30 minutes after incubation in the dark, the reaction was stopped with 2N

H_2SO_4. Absorbance was read at 450 nm using a Spectramax M2 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) and corrected for plate absorbance at 570 nm. Concentrations of cytokines in the samples were determined by interpolation from standard curves calculated for each plate using appropriate amounts of recombinant mouse cytokines.

Flow cytometry and intracellular cytokine staining

For intracellular cytokine staining, cells were stimulated with leukocyte activation cocktail with GolgiPlug (BD Pharmingen) at 2ul/10^6 cells, either immediately ex vivo or, in the case of cultured cells, after overnight culture in fresh medium without antigen. After 6 hours of stimulation, cells were harvested, stained with anti-CD4 PercP Cy5.5, fixed, permeabilized and subsequently stained with combinations of the following antibodies: PE-conjugated anti-mouse IL-4, APC-conjugated anti-mouse IL-10, PE-conjugated anti-mouse Foxp3 and FITC-conjugated anti-mouse IFN-γ (BD-Pharmingen). The cells were then analyzed using a LSR II Optical Bench flow cytometer and FACSDiva software (BD Biosciences).

A2A receptor agonist and inhibitor treatment

$CD4^+$ T cells from livers of infected wild type C57BL/6 mice were isolated as described above and incubated for 18 hours at 37°C in 5% carbon dioxide in TCM containing either 1uM CGS21680 (agonist), 1uM ZM241385 (inhibitor) or 1uM of both. After washing, $CD4^+$ cells were then cultured with $CD11c^+$ splenic dendritic cells from non-infected C57BL/6 mice in the presence of 50μg/ml SWAP or 20ug/ml SEA. Following incubation

for 72 h at 37°C in 5% carbon dioxide, supernatants were collected and immediately frozen at –80°C for subsequent determination of cytokine concentration.

Histology and fibrosis

Liver samples were fixed in 4 % neutral-buffered formalin and embedded in paraffin for sectioning. Liver sections were stained with picro-sirius red and evaluated under both bright field illumination and crossed polarizing filters. Using Sigma Scan Pro version 5, the area of the collagen fibers seen in a field of 200x magnification was determined. An arbitrary unit was given to the area: 10000 pixels equal 1 unit and the fiber count is reported as an average of the units.

Statistical analysis

Because data frequently exhibited unequal variance between experimental groups, differences between variables were evaluated by nonparametric unpaired *t* test utilizing Welch's correction in GraphPad Prism software version 4.0. $P < 0.05$ were considered significant. All experiments were performed at least twice with similar results.

Results

Production of IL-10 by hepatic $CD4^+$ T cells during pre-patent schistosome infection

To obtain data that would be most representative of cytokine production by $CD4^+$ T cells *in vivo,* we analyzed cytokine gene transcription by $CD4^+$ cells isolated by magnetic cell sorting from wild type mice at weekly intervals during the first 4 weeks of infection. Following infection by exposure of the tail skin to cercariae, $CD4^+$ cells were isolated at weekly intervals from nodes draining the site of infection (medial iliac and sacral LN), from nodes draining tissues that the migrating parasites traverse (tracheobronchial and mesenteric LN), and from the spleen and liver, as well as from a lymph node that is not associated with the migratory route of the schistosomula (inguinal LN). Transcript levels of IFN-γ, IL-4 and IL-10 were quantified by real-time PCR and compared to the levels detected in $CD4^+$ cells isolated from the same anatomic locations in non-infected mice. Consistent with reports by others (Angeli et al., 2001; Hogg et al., 2003a; Hogg et al., 2003b) , evidence of a mixed response characterized by transient expression of IFN-γ, IL-4 and IL-10 by $CD4^+$ cells was observed in nodes draining the site of infection one week post exposure, which then diminished by week 2 (Fig. 3A). Unexpectedly, in the other locations analyzed throughout the course of the infection, IFN-γ transcript levels in $CD4^+$ cells did not show any elevation above the levels detected in cells from non-infected animals (Fig. 3A). Comparable results were also obtained for IL-4 (Fig. 3A). However, beginning at week 1 post infection and coincident with the arrival of migrating parasites in the portal system, hepatic $CD4^+$ cells exhibited a dramatic increase in the expression of IL-10, in excess of 50-fold higher than the levels

found in hepatic $CD4^+$ cells isolated from non-infected mice (Fig. 3A). High levels of IL-10 transcript were sustained through the following weeks, reaching a peak in excess of 200-fold relative to non-infected levels by week 4 post infection (Fig. 3A).

To determine whether the relative abundance of IL-10 and IFN-γ transcripts in hepatic $CD4^+$ cells was reflected in the production of these two proteins by hepatic $CD4^+$ cells, $CD4^+$ cells were isolated by magnetic cell sorting from the livers of infected mice at 4 weeks post infection and their production of IFN-γ and IL-10 measured upon re-stimulation *in vitro* with dendritic cells and SWAP. In agreement with the relative abundance of the two cytokine transcripts, isolated hepatic $CD4^+$ from infected animals elaborated approximately 40-fold more IL-10 in response to SWAP than cells from non-infected animals, while levels of IFN-γ production by these cells were less than two-fold higher than the background levels of IFN-γ detected in this assay (Fig. 3B). Similar results were obtained when hepatic $CD4^+$ T cells were isolated by flow cytometric cell sorting to exclude $CD4^+$ myeloid and NKT cells (Fig. 3C), indicating that these cell types are not a major source of IL-10 when cells are isolated by magnetic methods. Thus $CD4^+$ T cells isolated from the site of antigen deposition during pre-patent schistosome infection predominantly produced IL-10 rather than IFN-γ or IL-4.

Hepatic IL-10-producing $CD4^+$ T cells are predominantly Foxp3-negative

To determine if IL-10-producing $CD4^+$ T cells are Foxp3-positive regulatory T cells (Treg), $CD4^+$ T cells were isolated from the livers of infected mice by flow cytometry, co-cultured with antigen and dendritic cells for 3 days and then analyzed for IL-10 and Foxp3 expression by flow cytometry. This analysis revealed that the majority

of IL-10-producing $CD4^+$ T cells elicited by either SWAP or anti-CD3 stimulation were Foxp3-negative (Fig. 4). Because there is evidence that Th1 $CD4^+$ T cells can constitute an important source of IL-10 during the response to infectious agents (Jankovic et al., 2007) and the response to schistosome worms had previously been characterized as a Th1 response (Pearce and MacDonald, 2002), we analyzed hepatic $CD4^+$ T cells for simultaneous production of IL-10 and IFN-γ. This analysis revealed that SWAP stimulation primarily induced IL-10 production and not IFN-γ production by hepatic $CD4^+$ T cells, and that hepatic $CD4^+$ T cells predominantly produced either one cytokine or the other, while only a minor proportion produced both IL-10 and IFN-γ (Fig. 4).

IL-10 regulates IFN-γ production but not IL-4 production by hepatic and splenic $CD4^+$ T cells during pre-patent schistosome infection

Because IL-10 plays a central role in the regulation of IFN-γ production by Th1 cells, we hypothesized that IL-10 regulates IFN-γ production by $CD4^+$ T cells during pre-patent schistosome infection. To test this hypothesis, we assessed the IFN-γ response of hepatic $CD4^+$ T cells to worm antigens in IL-$10^{-/-}$ mice and wild type mice at 4 weeks post-infection. Upon stimulation with SWAP and wild type dendritic cells, hepatic $CD4^+$ T cells from infected IL-$10^{-/-}$ mice produced high levels of IFN-γ as compared to $CD4^+$ T cells from wild type mice (Fig. 5A), suggesting that IL-10 regulates IFN-γ production by $CD4^+$ T cells in response to schistosome worm antigens. In contrast, hepatic $CD4^+$ T cells from wild type and IL-$10^{-/-}$ mice produced comparable levels of IL-4 in response to SWAP (Fig. 5A), suggesting that IL-10 does not regulate Th2 responses to schistosome worms. Finally, we also measured IL-10 levels in co-cultures of wild type dendritic cells

and IL-10$^{-/-}$ CD4^{+} T cells, to determine whether dendritic cells contributed to the level of IL-10 detected in the supernatant. Only background levels of IL-10 were detected (Fig. 5A), demonstrating that the IL-10 produced in these co-cultures is of CD4^{+} T cell origin.

Previous analyses of CD4^{+} T cell responses to the early stages of schistosome infection have relied on *in vitro* recall responses to worm antigens by unfractionated splenocyte populations to examine effector T cell phenotype. These analyses have mostly reported low levels of IFN-γ production by splenocyte cultures and have led to the characterization of the CD4^{+} T cell response at this stage of infection as a modest Th1 response. Because we did not find consistently elevated levels of IFN-γ transcript in splenic CD4^{+} T cells immediately ex vivo (Fig. 3A), we hypothesized that splenic CD4^{+} T cells do not express IFN-γ *in vivo* but can be induced to do so *in vitro* upon stimulation with antigen. Alternatively, production of IFN-γ by other cells such as CD8^{+} T cells might account for the discrepancy between our data and those previously published. To discriminate between these possibilities, we isolated splenic CD4^{+} T cells and tested their ability to produce IFN-γ and other cytokines when stimulated *in vitro* with SWAP and dendritic cells. In agreement with previously published results, and in stark contrast to hepatic CD4^{+} T cells, splenic CD4^{+} T cells from wild type mice produced high levels of IFN-γ *in vitro* (Fig. 5B). However, in parallel with hepatic CD4^{+} T cells, IL-10 appeared to be critical for regulating this IFN-γ production, as CD4^{+} T cells from infected IL-10$^{-/-}$ mice produced threefold more IFN-γ in response to worm antigens than did CD4^{+} T cells from infected wild type mice (Fig. 5B). Interestingly, splenic CD4^{+} T cells from infected animals also produced low levels of IL-4 in response to worm antigens but this was not regulated by IL-10, as CD4^{+} T cells from wild type and IL-10$^{-/-}$ mice produced similar

levels of IL-4 (Fig. 5B). Consistent with a role for IL-10 in regulating IFN-γ production by $CD4^+$ T cells in the spleen, wild type splenic $CD4^+$ T cells readily produced IL-10 upon restimulation with antigen and dendritic cells *in vitro* (Fig 3B). Thus, while expression of IFN-γ and IL-10 message by splenic $CD4^+$ T cells was barely detectable immediately ex vivo (Fig. 3A), these cells readily produced both cytokines upon restimulation *in vitro* (Fig. 5B), suggesting that primed $CD4^+$ T cells with specificity for schistosome worm antigens reside in the spleen but, in contrast to hepatic $CD4^+$ T cells, do not actively produce either cytokine *in vivo*.

Vaccination overcomes regulation of IFN-γ production by hepatic $CD4^+$ T cells during pre-patent schistosome infection

Previous studies have shown that vaccination of experimental animals by exposure to radiation-attenuated cercariae induces immunity to subsequent challenge with non-irradiated cercariae (Hewitson et al., 2005). Furthermore, there is clear evidence that this irradiated cercaria vaccine, in contrast to non-irradiated cercariae, induces a vigorous Th1 response to worm antigens that mediates protection to subsequent challenge, by activating macrophages to kill migrating schistosomula (James et al., 1984). We therefore hypothesized that differential induction of IL-10 by non-irradiated and irradiated parasites might account for the difference in the magnitude of the Th1 response to each – substantial IL-10 production in the former preventing development of an effective Th1 response, and diminished production in the latter allowing for development of a vigorous and protective Th1 response. To test this hypothesis, we examined IFN-γ and IL-10 production by $CD4^+$ T cells from infected animals that either

had or had not been vaccinated previously. Hepatic $CD4^+$ T cells from vaccinated mice produced very high levels of IFN-γ (Fig. 5C) while, as noted previously (Fig. 3), cells from animals that had not been vaccinated did not produce IFN-γ (Fig. 5C). However, hepatic $CD4^+$ T cells from vaccinated and non-vaccinated mice produced comparable levels of IL-10 (Fig. 5C) suggesting that, while vaccination facilitates expression of IFN-γ, this is not because IL-10 expression is inhibited as a result of vaccination. Rather, vaccination appears to overcome the inhibitory effects if IL-10, allowing IFN-γ to be expressed despite production of IL-10.

Adenosine signaling through A2AR regulates cytokine expression by hepatic $CD4^+$ T cells during pre-patent schistosome infection

As IL-10 plays a significant regulatory role in the $CD4^+$ T cell response to schistosome antigens during pre-patent infection (Fig. 5A and 5B), we investigated whether other regulatory mechanisms also influence this response. Recently, adenosine signaling through the A2A receptor was shown to play a significant role in regulating the production of IFN-γ by T cells, particularly in the liver (Yang et al., 2006). We therefore examined schistosome-induced hepatic $CD4^+$ T cell responses in $A2AR^{-/-}$ mice. When cytokine mRNA levels in $CD4^+$ T cells from 4 week-infected mice were determined, cells from $A2AR^{-/-}$ mice contained significantly higher levels of IFN-γ and IL-4 transcripts than cells from wild type mice, while IL-10 transcript levels were similar (Fig. 6A), suggesting that expression of both Th1 and Th2 cytokines are regulated by A2AR expression. To evaluate whether this dysregulation extended to the production of cytokine protein, hepatic $A2AR^{-/-}$ $CD4^+$ T cells were stimulated *in vitro* with schistosome

antigens and wild type dendritic cells. A2AR$^{-/-}$ CD4^{+} T cells produced more IFN-γ than wild type cells stimulated under identical conditions, while production of IL-4 and IL-10 were similar (Fig. 6B), suggesting that dysregulation due to loss of A2AR predominantly affected production of IFN-γ at the protein level. Because A2AR signaling has been implicated in the inhibition of IL-12 production by antigen-presenting cells, we investigated whether the increased propensity of A2AR$^{-/-}$ CD4^{+} T cells to produce IFN-γ was due to induction of higher IL-12 levels by schistosome infection in A2AR$^{-/-}$ mice. However, no differences in IL-12 transcript levels in livers of infected wild type and A2AR$^{-/-}$ mice were detected (Fig. 6C). To directly test whether A2AR$^{-/-}$ dendritic cells stimulate higher levels of IFN-γ production by CD4^{+} T cells primed by schistosome infection, hepatic CD4^{+} T cells were isolated from infected wild type mice and stimulated *in vitro* with either wild type or A2AR$^{-/-}$ dendritic cells. Regardless of the source of the dendritic cells, wild type CD4^{+} T cells produced similar levels of IFN-γ, IL-4 and IL-10 (Fig. 6D), suggesting that the increased IFN-γ production by A2AR$^{-/-}$ CD4^{+} T cells is due to dysregulation of cytokine production at the level of the T cell itself, rather than due to loss of A2AR expression on dendritic cells.

Direct modulation of hepatic CD4^{+} T cell cytokine production by A2AR signaling

We first examined the level of A2AR expression in hepatic CD4^{+} T cells relative to whole liver tissue at 4 weeks post infection. By quantitative real-time PCR, hepatic CD4^{+} T cells were found to express approximately ten-fold more A2AR messenger RNA compared to whole liver tissue (Fig. 7A), suggesting this receptor is preferentially expressed by CD4^{+} T cells. Furthermore, when A2AR expression by CD4^{+} T cells from

livers of infected and non-infected mice were compared, A2AR expression by hepatic $CD4^+$ T cells was induced approximately sixty-fold by schistosome infection (Fig. 7B), suggesting that schistosome worms selectively induce A2AR expression by hepatic $CD4^+$ T cells due to increase in adenosine levels in the liver as a result of inflammatory responses (Deaglio et al., 2007).

To test whether cytokine production by hepatic $CD4^+$ T cells was directly regulated by A2AR signaling in T cells themselves, $CD4^+$ T cells were isolated from the livers of 4 week-infected wild type mice, pre-treated with A2AR agonist, A2AR inhibitor or a combination of both, and then tested for their ability to make cytokines in response to schistosome antigens presented by non-treated dendritic cells. Consistent with the data from $A2AR^{-/-}$ mice (Fig. 7B), pharmacologic inhibition of A2AR on hepatic $CD4^+$ T cells significantly increased the production of IFN-γ (Fig. 7C). Conversely, treatment with A2AR agonist caused a significant decrease in IFN-γ production, and treatment with both agonist and inhibitor resulted in an intermediate level of IFN-γ production (Fig. 7C). IL-4 production was modulated in a broadly similar but less pronounced pattern, with significant differences in cytokine production evident between inhibitor-treated and non-treated cells and between agonist- and inhibitor-treated cells (Fig. 7D). Production of IL-10 by hepatic $CD4^+$ T cells was modulated in an identical manner to IFN-γ by agonist, inhibitor and combined agonist-inhibitor treatments (Fig. 7E).

A2AR modulates Th2-mediated pathology during patent schistosome infection

The data in Figs. 6 and 7 suggest that, in a regulatory immunological milieu, A2AR signaling in hepatic $CD4^+$ T cells primarily modulates production of IFN-γ. To test whether A2AR signaling can also modulate Th2 responses, we assessed the role of A2AR signaling in schistosome egg-induced liver fibrosis, a Th2-mediated process in which IL-13 plays a central role in the stimulation of fibrosis. To assess the extent of fibrosis, sections of liver tissue from wild type and $A2AR^{-/-}$ mice were compared by staining with picrosirius red, a substance that specifically stains collagen fibers. Tissue from non-infected wild type (Fig. 8A) and $A2AR^{-/-}$ mice (Fig. 8B) stained minimally with picrosirius red, revealing a normal amount of collagen in non-infected animals of both strains. After 8 weeks infection with *S. mansoni*, both strains exhibited substantial amounts of collagen in the liver, in association with egg-induced granulomas (Fig. 8C, 8D). When viewed under bright-field illumination, livers of infected $A2AR^{-/-}$ mice (Fig. 8D) contained more red-staining collagen than livers of wild type mice (Fig. 8C). When picrosirius red-stained liver sections from infected animals were examined through crossed polarizing filters, $A2AR^{-/-}$ mice again displayed greater amounts of birefringent collagen fibers (Fig. 8F) than wild type mice (Fig. 8E). Quantitation of collagen levels from digital micrographs revealed that the increased level of fibrosis in $A2AR^{-/-}$ mouse livers was statistically significant ($P = 0.0009$; Fig. 8G). Thus, loss of A2AR expression led to exacerbated egg-induced fibrosis.

A2AR expression modulates production of type 2 cytokines by $CD4^+$ T cells

To determine whether the exacerbated fibrosis observed in infected $A2AR^{-/-}$ mice was due to dysregulation of type 2 cytokine production by $CD4^+$ T cells, $CD4^+$ T cell responses were assessed by examining cytokine gene expression and synthesis immediately ex vivo by real-time PCR and intracellular cytokine staining. At 8 weeks post infection, real-time PCR analysis of cytokine transcript levels in purified splenic $A2AR^{-/-}$ $CD4^+$ T cells immediately ex vivo revealed significant increases in IFN-γ, IL-4, IL-10 and IL-13 gene expression when compared to $CD4^+$ T cells from wild type mice (Fig. 9A), while IL-5 expression was similar in both genotypes. Likewise, when cytokine synthesis by $CD4^+$ T cells was assessed immediately ex vivo by intracellular cytokine staining, increases in the number of cells producing IFN-γ, IL-4 and IL-10 were detected in $A2AR^{-/-}$ mice compared to wild type mice (Fig. 9B), and the mean fluorescence intensity of the cytokine-producing $A2AR^{-/-}$ cells was higher for IL-4 and IL-10 when compared to wild type (Fig. 9B), suggesting that production of these cytokines was elevated. We also assessed cytokine production by wild type and $A2AR^{-/-}$ $CD4^+$ T cells by examining *in vitro* recall responses to schistosome egg antigens. When restimulated *in vitro* with wild type dendritic cells and SEA, splenic $CD4^+$ T cells from $A2AR^{-/-}$ mice showed a modest but significant increase in the recall IFN-γ response compared to cells from wild type animals, while recall responses of the type 2 cytokines IL-4, IL-5 and IL-13, and the regulatory cytokine IL-10 were not significantly altered in this assay (Fig. 9C). Thus, A2AR-mediated modulation of type 2 cytokine production by $CD4^+$ T cells was evident when responses were assessed immediately ex vivo, but not upon restimulation with antigen *in vitro*.

Direct A2AR-mediated modulation of type 2 cytokine production by $CD4^+$ T cells

To determine whether A2AR signaling in Th2 cells can directly modulate production of type 2 cytokines, we first assessed the expression of A2AR by splenic $CD4^+$ T cells. At 8 weeks post infection, splenic $CD4^+$ T cells from wild type mice expressed levels of A2AR mRNA that were comparable to those expressed by hepatic $CD4^+$ T cells at 4 weeks post infection (Fig. 10A). Thus schistosome infection caused induction of A2AR expression on splenic $CD4^+$ T cells by 8 weeks post infection. Next, we assessed the responsiveness of splenic $CD4^+$ T cells to A2AR agonist and inhibitor *in vitro*. When stimulated with egg antigens, pre-treatment with agonist or inhibitor caused significant reduction or enhancement of IFN-γ production, respectively, while pre-treatment with both agonist and inhibitor produced an intermediate effect (Fig. 10B). For type 2 cytokines, pre-treatment with inhibitor produced modest but significant increases in IL-4 (Fig. 10C), IL-10 (Fig. 10D) and IL-13 (Fig. 10E) production when compared to cells treated with agonist. However, when compared to non-treated cells, these increases were only significant for IL-4 (Fig. 10C) and IL-10 (Fig. 10D) and not for IL-13 (Fig. 10E). Furthermore, in contrast to IFN-γ production, treatment with agonist did not modulate production of IL-4, IL-10 or IL-13 compared to untreated cells (Fig. 10C-E). Taken together, our results show that type 2 cytokine production by $CD4^+$ T cells can be directly modulated by A2AR signaling *in vitro*, but that this modulation is less pronounced than the modulation of IFN-γ production.

Discussion

Previous studies have shown that the early, pre-patent phase of schistosome infection is characterized by a modest and transient systemic Th1 response to worm antigens that is subsequently replaced at 6 weeks post infection by a dominant Th2 response to egg antigens (Pearce et al., 1991; Pearce and MacDonald, 2002). The data we present here provide an explanation for the absence of robust responses to schistosome worms during a primary infection, as we show that infection with cercariae rapidly establishes a systemic regulatory $CD4^+$ T cell response (Fig. 3) that strongly modulates concomitant pro-inflammatory responses to worm antigens (Fig. 5). Indeed, our data suggest that, by 4 weeks post infection, IL-10 is the predominant cytokine made by $CD4^+$ T cells *in vivo* (Fig. 3A). In demonstrating the establishment of a systemic regulatory response, our results are in agreement with previously published studies which showed that schistosome larvae induce local expression of IL-10 in the skin at the site of infection (Angeli et al., 2001; Hogg et al., 2003a; Hogg et al., 2003b). Several potential mechanisms by which schistosomula induce IL-10 expression have been proposed, including the production of parasite-derived prostaglandins (Ramaswamy et al., 2000) and proteins with anti-inflammatory properties (Rao and Ramaswamy, 2000). Here we show that the IL-10 response to larval schistosomes rapidly extends to the liver, coincident with the arrival of the migrating larvae in the portal circulation one week post infection (Fig. 3A)(Georgi et al., 1986). The rapid and widespread induction of IL-10 by schistosome larvae may contribute to the inability of the immune system to mediate protection against incoming schistosomula. Indeed, IL-10 has been shown to play an

important role in inhibiting IL-12-mediated priming of protective Th1 responses to schistosome antigens (Hogg et al., 2003b).

Previous studies on the later stages of schistosome infection have shown that $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells are an important source of the IL-10 produced in response to egg antigens (McKee and Pearce, 2004). However, our data suggest that the hepatic IL-10-producing $CD4^+$ T cells induced by schistosome worms do not express Foxp3 (Fig. 4) or CD25 (data not shown). Indeed, the liver contains relatively few $Foxp3^+$ $CD4^+$ T cells, comprising less than one percent of the total $CD4^+$ T cell population (Fig. 4). Rather, our data suggest that schistosome worm-induced IL-10 producers represent a population of inducible regulatory T cells similar to Tr1 or Th3 cells (Mills and McGuirk, 2004). Alternatively, schistosome worm-induced IL-10-producing $CD4^+$ T cells may represent a population of IL-10-expressing Th1 cells, similar to those induced by other parasites such as *Toxoplasma* (Jankovic et al., 2007) and *Leishmania* (Anderson et al., 2007; Nylen et al., 2007). However, only a minority of the hepatic IL-10-producing cells elicited by schistosome worms simultaneously expressed IFN-γ, while the majority expressed IL-10 alone (Fig. 4). Thus the relationship of these cells to IL-10-producing Th1 cells remains unclear.

Consistent with previously published results on cercaria-infected skin (Hogg et al., 2003b), worm-induced IL-10 played a significant role in suppressing concomitant Th1 responses, as hepatic $CD4^+$ T cells from IL-$10^{-/-}$ mice exhibited high levels of IFN-γ production (Fig. 5A). In contrast, IL-10 had little effect on the low levels of IL-4 produced by hepatic $CD4^+$ T cells at 4 weeks post infection (Fig. 5A). Similar IL-10-mediated regulation of IFN-γ production by $CD4^+$ T cells was evident in the spleens of

infected mice at 4 weeks post infection (Fig 5B). However, in contrast to hepatic $CD4^+$ T cells, splenic $CD4^+$ T cells did not express detectable levels of IL-10 mRNA immediately ex vivo (Fig. 3A), suggesting this cytokine is not made *in vivo* but can be elicited by subsequent stimulation with schistosome antigens *in vitro*. Thus our data suggest that at 4 weeks post infection, the spleen contains primed schistosome-specific $CD4^+$ T cells that can produce IL-10 on encountering antigen but, in contrast to hepatic $CD4^+$ T cells, do not express IL-10 *in vivo*. The relative abundance of secreted and excreted worm antigens in the liver (Homewood et al., 1972; Stenger et al., 1967) compared to the spleen during early infection may account for this difference in T cell cytokine expression.

Previously published data have shown that vaccination with irradiated schistosome cercariae induces robust Th1 responses that mediate protection against subsequent challenge with non-irradiated cercariae (James et al., 1987). Because our data show that primary infection with normal cercariae induces significant levels of IL-10 that modulate IFN-γ production, we questioned whether vaccination resulted in robust Th1 responses because exposure to irradiated cercariae interferes with the ability of vaccinated mice to produce IL-10 upon challenge. However, our results clearly show that vaccinated mice produce high levels of both IFN-γ and IL-10 upon challenge (Fig. 5C). Thus, vaccination appears to release schistosome worm-induced Th1 responses from regulation by IL-10, even though the IL-10 response to schistosome larvae remains intact.

Because early Th cell responses to schistosome larvae in the liver appear to be heavily regulated by IL-10, we explored whether other regulatory mechanisms may also be involved in modulating $CD4^+$ T cell responses to schistosome infection. One regulatory mechanism of considerable importance in the liver which has not been

evaluated in the context of schistosome infection is the anti-inflammatory effects of adenosine signaling through the A2AR. A2AR signaling modulates Th1 responses by two mechanisms. First, engagement of A2AR on dendritic cells reduces IL-12 production (Hasko and Cronstein, 2004), leading to reduced IFN-γ production by $CD4^+$ T cells. Second, activation of A2ARs on $CD4^+$ T cells themselves appears to modulate IFN-γ production, as was recently shown in a model of ischaemia-reperfusion injury (Yang et al., 2006). Our data clearly demonstrate that A2AR signaling also modulates IFN-γ production during schistosome infection, both during the early regulatory response to worm antigens and during the later Th2 response to egg antigens. Furthermore, our data suggest this modulation is likely accomplished by the direct activation of A2AR on $CD4^+$ T cells, as evidenced by the responsiveness of IFN-γ production by $CD4^+$ T cells to A2AR agonists and inhibitors. Interestingly, production of IL-4, IL-13 and IL-10 by $CD4^+$ T cells during helminth infection was also modulated by A2AR signaling. However, in contrast to IFN-γ production, modulation of Th2 and regulatory cytokine production was most evident in $CD4^+$ T cells immediately ex vivo and was less pronounced upon restimulation with antigen *in vitro*, even after treatment with A2AR agonists and inhibitors. These differences suggest that modulation of Th2 and regulatory cytokines by A2AR is achieved by different mechanisms than Th1 cytokines, such as through activation of A2AR on antigen-presenting cells.

Contrary to expectations, our data show that Th2 cytokine production was augmented in the absence of A2AR signaling. Indeed, we found no evidence of a reciprocal decrease in Th2 cytokine expression in the face of elevated Th1 cytokine production. Importantly, loss of A2AR expression exacerbated levels of schistosome egg-

induced fibrosis – a Th2-mediated pathology primarily driven by production of IL-13 (Wynn, 2004; Wynn et al., 2004). Thus A2AR signaling appears to play an important and non-redundant role in the modulation of Th2 responses and their associated pathology, at least during schistosome infection, and argue that stimulation of A2AR signaling rather than its inhibition would be of greater benefit in alleviating the fibrosis associated with this debilitating and neglected tropical disease.

Figure 1: Hepatic $CD4^+$ T cells express IL-10 during early *S. mansoni* infection. A) $CD4^+$ T cells from lymph nodes, spleens and livers of infected WT mice at different time points (1-4 weeks), were isolated by MACS separation columns and cytokine mRNA levels from extracted RNA was measured by Real-time PCR. Fold expression of IFN-γ, IL-4 and IL-10 cytokines was normalized to mRNA cytokine levels from uninfected mice. B) Hepatic $CD4^+$ T cells isolated by MACS separation columns from livers of non-infected WT mice and 4 weeks infected WT mice were co-cultured with splenic dendritic cells from non-infected WT mice at a ratio of 1:10 ($0.5x10^6$ T cells: 50,000 DC's) and stimulated (■) or not stimulated (□) with *S. mansoni* worm antigen (Sm Ag;SWAP) for 3 days. IFN-γ and IL-10 cytokine protein levels from supernatants of infected, non-infected and DC's cultured alone (250,000 DC's), were measured by ELISA. C) Hepatic $CD4^+$ T cells isolated by FACS cell sorter from livers of non-infected WT mice and 4 weeks infected WT mice were co-cultured with splenic dendritic cells from non-infected WT mice at a ratio of 1:10 ($0.5x10^6$ T cells: 50,000 DC's) and stimulated (■) or not stimulated (□) with *S. mansoni* worm antigen (Sm Ag;SWAP) for 3 days. IFN-γ and IL-10 cytokine protein levels from supernatants were measured by ELISA.

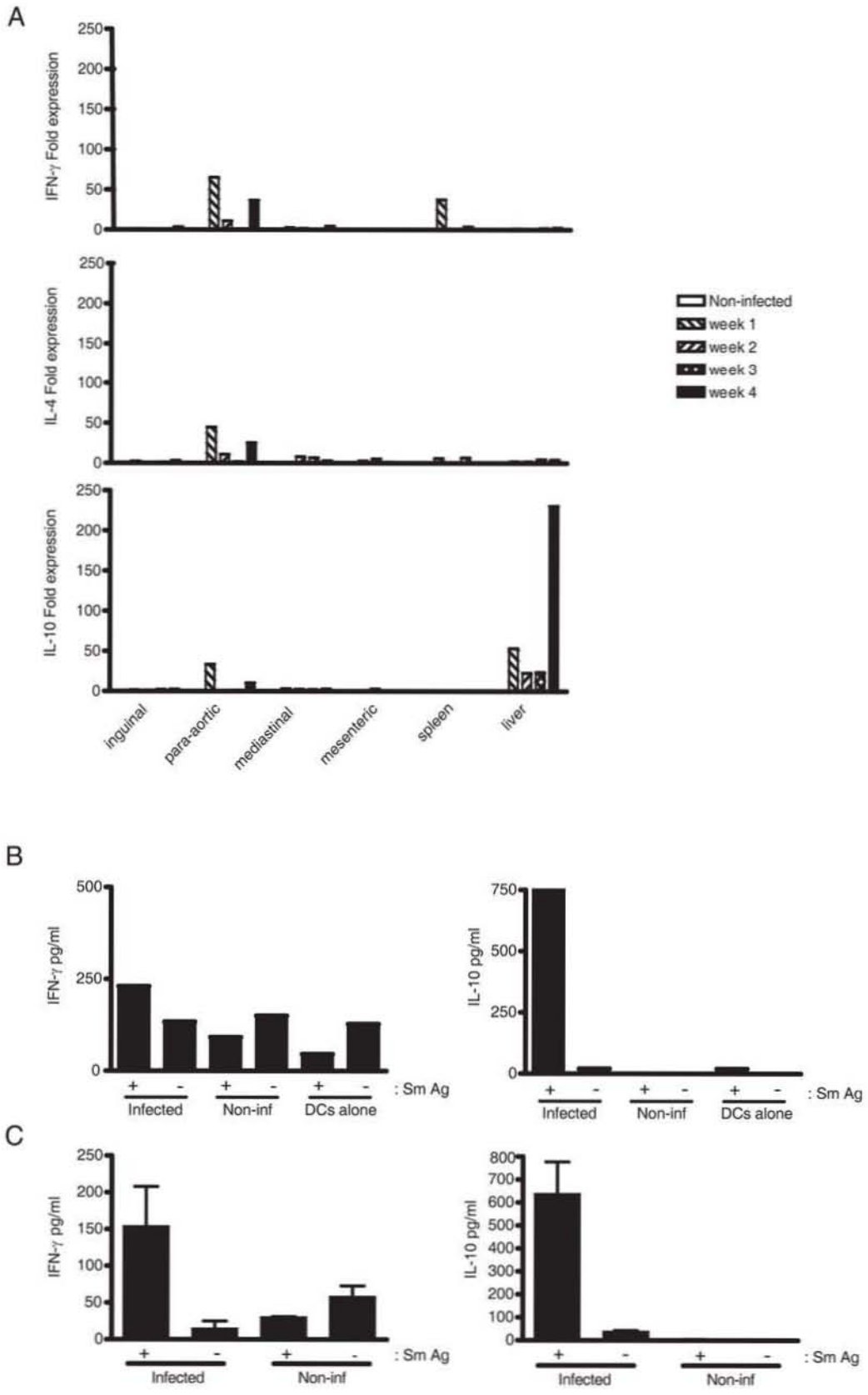

A
IFN-γ Fold expression
IL-4 Fold expression
IL-10 Fold expression
250
200
150
100
50
0
Non-infected
week 1
week 2
week 3
week 4
inguinal
para-aortic
mediastinal
mesenteric
spleen
liver
B
IFN-γ pg/ml
500
250
0
IL-10 pg/ml
750
500
250
0
+ - + - + - : Sm Ag
Infected
Non-inf
DCs alone
C
IFN-γ pg/ml
250
200
150
100
50
0
IL-10 pg/ml
800
700
600
500
400
300
200
100
0
+ - + - : Sm Ag
Infected
Non-inf

Figure 2: Hepatic $CD4^+$ T cells express IL-10 in early infection and are not $Foxp3^+$. Hepatic $CD4^+$ T cells from livers of 4 weeks infected WT mice were stained with $CD4^+$ PercP Cy5.5 and with NK1.1 PE for NKT cells and also other markers (CD8, B220, CD11c and CD11b). $CD4^+$ T cells that stained only with PercP Cy5.5 were collected by a FACS cell sorter, co-cultured with splenic dendritic cells from non-infected WT mice at a ratio of 1:10 ($0.5x10^6$ T cells: 50,000 DC's) and non-stimulated, stimulated with SWAP or anti-CD3 for 3 days. Cultured cells were rested over night, and stimulated with leukocyte activation cocktail for 6 hrs. Cells from non-stimulated, stimulated with SWAP, or stimulated with anti-CD3 positive control, were harvested, stained with anti-CD4 PercP Cy5.5, fixed, permeabilized and stained with APC-conjugated anti-mouse IL-10, PE-conjugated anti-mouse Foxp3 and FITC-conjugated anti-mouse IFN-γ. Cells were analyzed using a LSR II Optical Bench flow cytometer. Numbers are percentages of total gated $CD4^+$ T cells. Results were representative of two separate experiments.

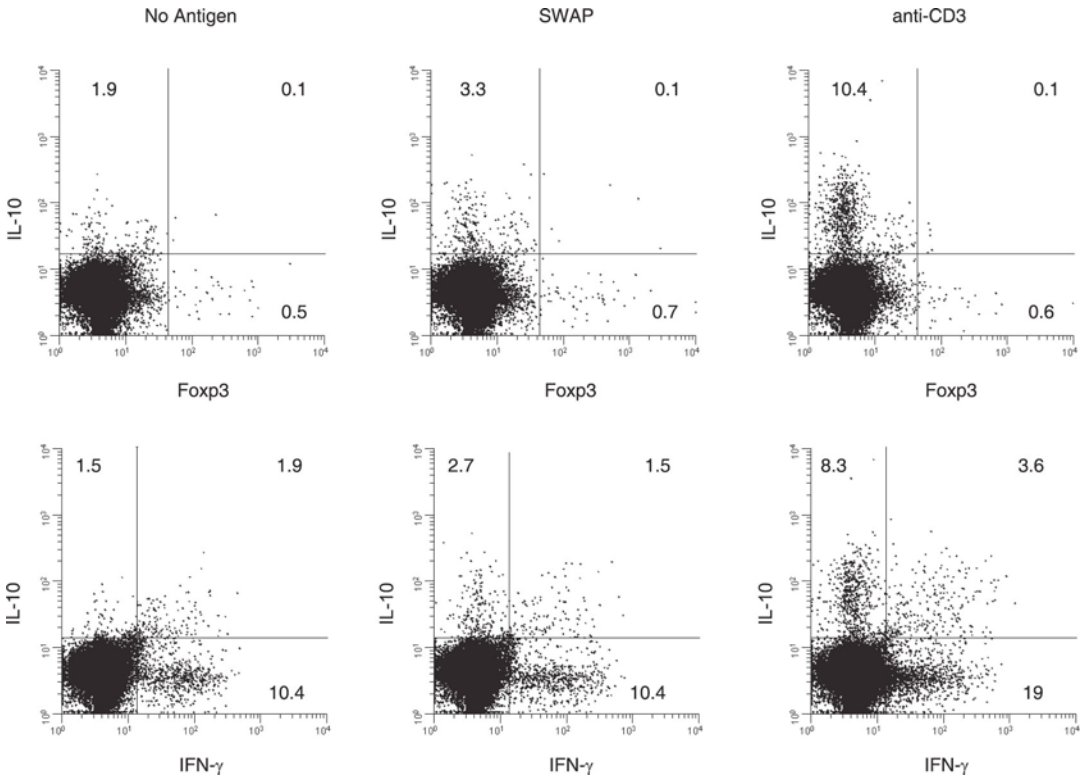

No Antigen
SWAP
anti-CD3
1.9
0.1
0.5
3.3
0.1
0.7
10.4
0.1
0.6
IL-10
Foxp3
1.5
1.9
10.4
2.7
1.5
10.4
8.3
3.6
19
IFN-γ

Figure 3: Regulatory response in early *S. mansoni* infection inhibits IFN-γ production. A) Hepatic and B) splenic $CD4^+$ T cells from 4 weeks infected WT and IL-$10^{-/-}$ mice were isolated by MACS separation columns. Cells were co-cultured with splenic dendritic cells from non-infected WT mice at a ratio of 1:10 ($2x10^6$ T cells/ml:200,000 DC's) and stimulated with SWAP (■) or not stimulated (□) for 3 days. IFN-γ, IL-4, and IL-10 cytokine protein levels were measured by ELISA. C) Hepatic $CD4^+$ T cells from 4 weeks infected WT or 3x vaccinated mice were isolated by MACS separation columns and co-cultured with splenic dendritic cells from non-infected WT mice at a ratio of 1:10 ($0.5x10^6$ T cells: 50,000 DC's) and stimulated with SWAP (■) or not-stimulated (□) for 3 days. IFN-γ and IL-10 cytokine protein levels were measured by ELISA.

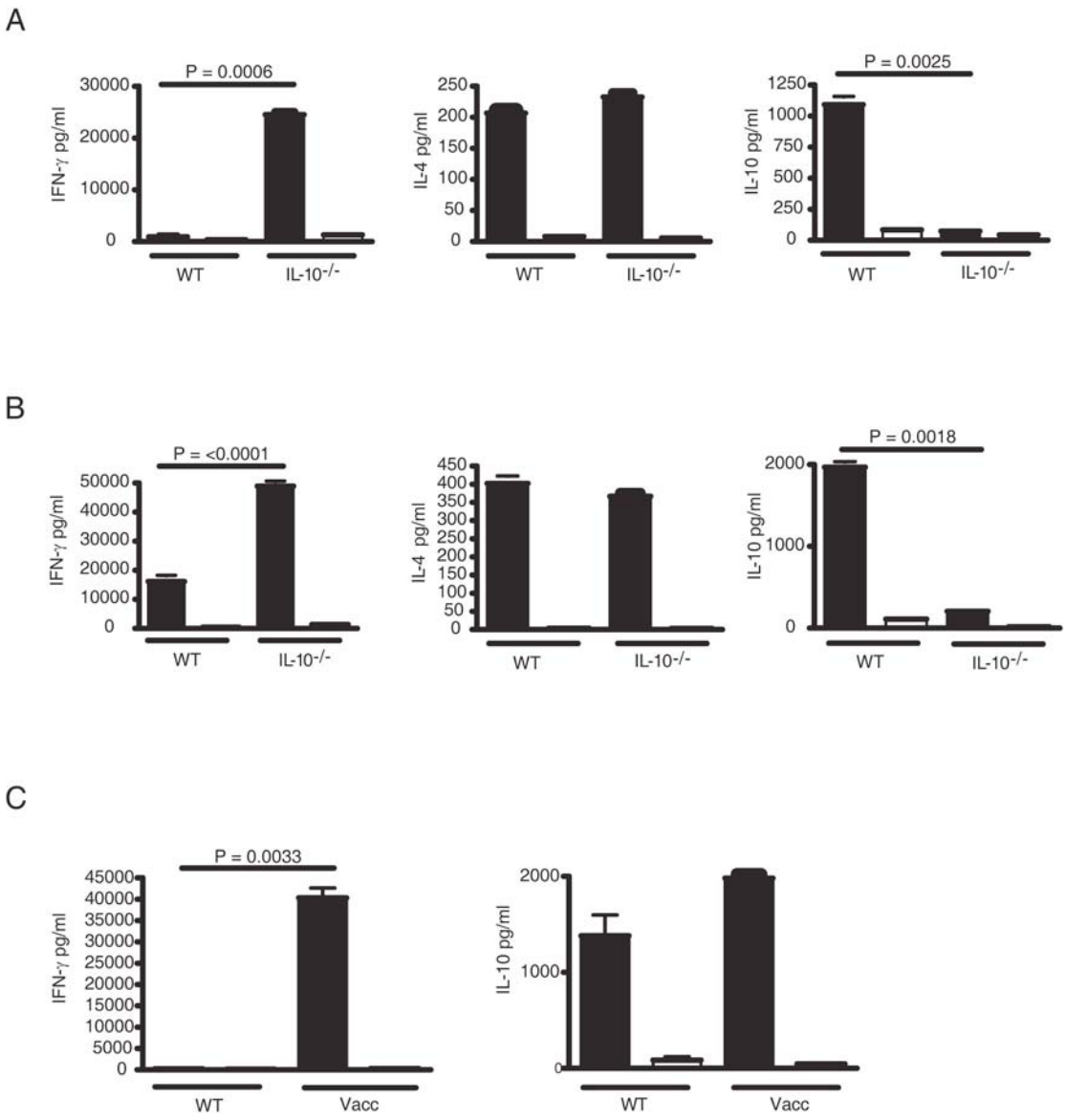

A
P = 0.0006
IFN-γ pg/ml
30000
20000
10000
0
WT
IL-10-/-
IL-4 pg/ml
250
200
150
100
50
0
WT
IL-10-/-
P = 0.0025
IL-10 pg/ml
1250
1000
750
500
250
0
WT
IL-10-/-
B
P = <0.0001
IFN-γ pg/ml
50000
40000
30000
20000
10000
0
WT
IL-10-/-
IL-4 pg/ml
450
400
350
300
250
200
150
100
50
0
WT
IL-10-/-
P = 0.0018
IL-10 pg/ml
2000
1000
0
WT
IL-10-/-
C
P = 0.0033
IFN-γ pg/ml
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
WT
Vacc
IL-10 pg/ml
2000
1000
0
WT
Vacc

Figure 4: Adenosine signaling through the A2A receptor regulates IFN-γ during early *S.mansoni* infection. A) Hepatic $CD4^+$ T cells from 4 weeks infected WT and $A2AR^{-/-}$ mice were isolated by MACS separation columns and cytokine mRNA levels from extracted RNA was measured by Real-time PCR. Fold expression of IFN-γ, IL-4 and IL-10 cytokines was normalized to mRNA cytokine levels from infected WT mice. B) Hepatic $CD4^+$ T cells from 4 weeks infected WT and $A2AR^{-/-}$ mice were isolated by MACS separation columns and co-cultured with splenic dendritic cells from non-infected WT mice at a ratio of 1:10 ($0.5x10^6$ T cells: 50,000 DC's) and stimulated with SWAP(■) or not stimulated (□) for 3 days. IFN-γ, IL-4 and IL-10 cytokine protein levels were measured by ELISA. C) RNA from whole liver tissues of 4 weeks infected WT and $A2AR^{-/-}$ mice were extracted and cytokine mRNA levels was measured by Real-time PCR. Fold expression of IL-12 cytokine from $A2AR^{-/-}$ mice was normalized to mRNA cytokine levels from infected WT mice. n = 3 for each group. D) Isolated hepatic $CD4^+$ T cells from 4 weeks infected WT mice co-cultured with either splenic dendritic cells from non-infected WT mice or splenic dendritic cells from non-infected $A2AR^{-/-}$ mice ($0.5x10^6$ T cells: 50,000 DC's). Cultured cells were stimulated with SWAP (■) or not stimulated (□) for 3 days and IFN-γ, IL-4 and IL-10 cytokine protein levels were measured by ELISA.

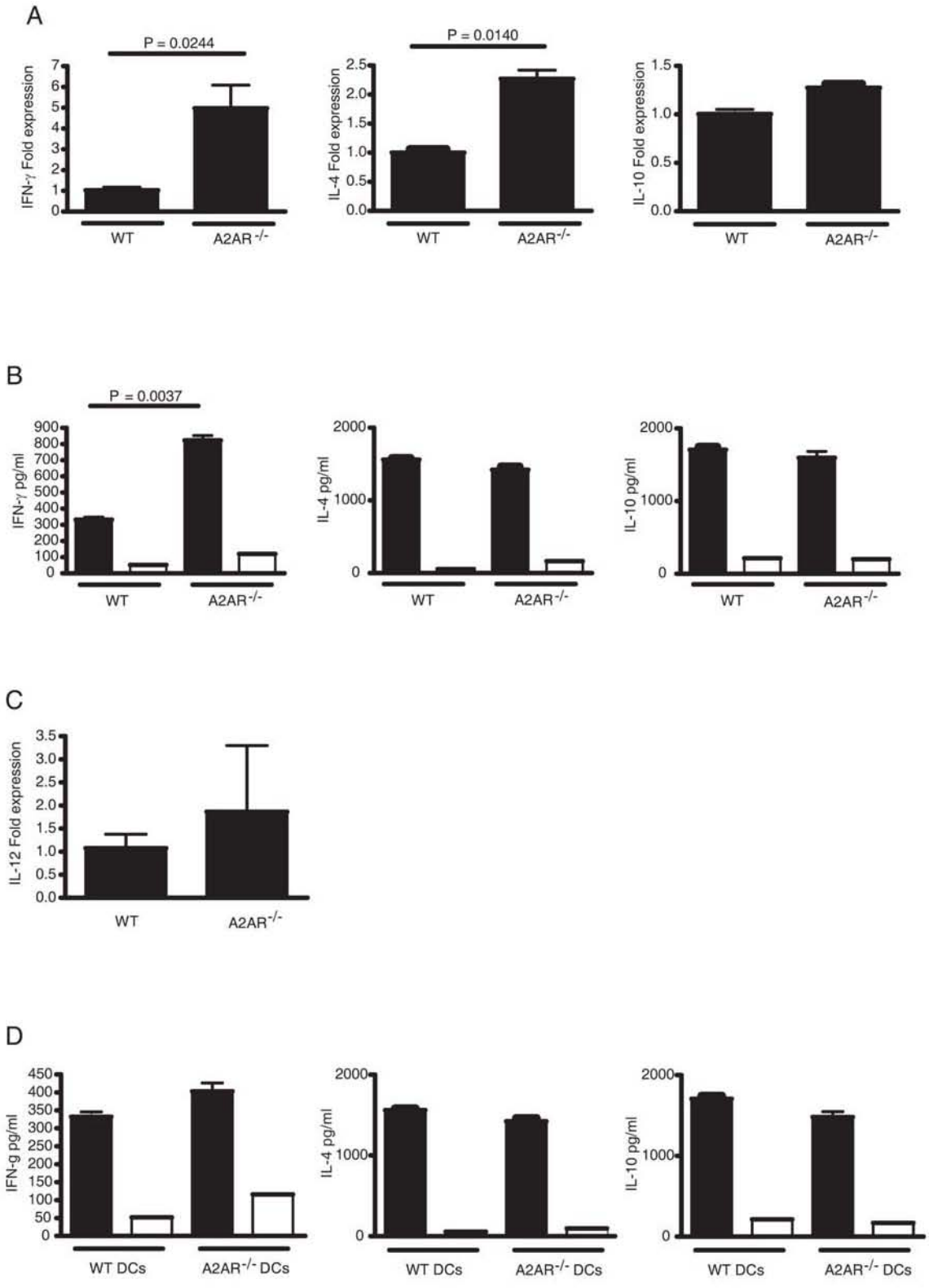

A
P = 0.0244
IFN-γ Fold expression
WT
A2AR-/-
P = 0.0140
IL-4 Fold expression
IL-10 Fold expression
B
P = 0.0037
IFN-γ pg/ml
IL-4 pg/ml
IL-10 pg/ml
C
IL-12 Fold expression
D
IFN-g pg/ml
WT DCs
A2AR-/- DCs

Figure 5: Signaling through the A2A receptor directly affects $CD4^+$ T cell responses in early *S. mansoni* infection. A) RNA from hepatic $CD4^+$ T cells of 4 weeks infected WT mice were extracted and A2AR mRNA levels was measured by Real-time PCR. Fold expression of A2AR was normalized to A2AR mRNA levels from whole liver tissues of 4 weeks infected WT mice. B) A2AR mRNA levels from hepatic $CD4^+$ T cells of 4 weeks infected WT mice was measured by Real-time PCR, and fold expression was normalized to A2AR mRNA levels from hepatic $CD4^+$ T cells of non-infected WT mice. Hepatic $CD4^+$ T cells from 4 weeks infected C57 BL/6 WT mice were isolated and incubated for 18 hrs treated with either 1 uM A2AR Agonist (CGS), 1 uM A2AR Inhibitor (ZM), 1 uM Agonist + 1 uM Inhibitor, or not treated. Cells were then co-cultured with splenic dendritic cells from non-infected WT mice ($1x10^6$ T cells:100,000 DC's) and stimulated with SWAP for 3 days. C) IFN-γ, D) IL-4 and E) IL-10 protein levels were measured by ELISA.

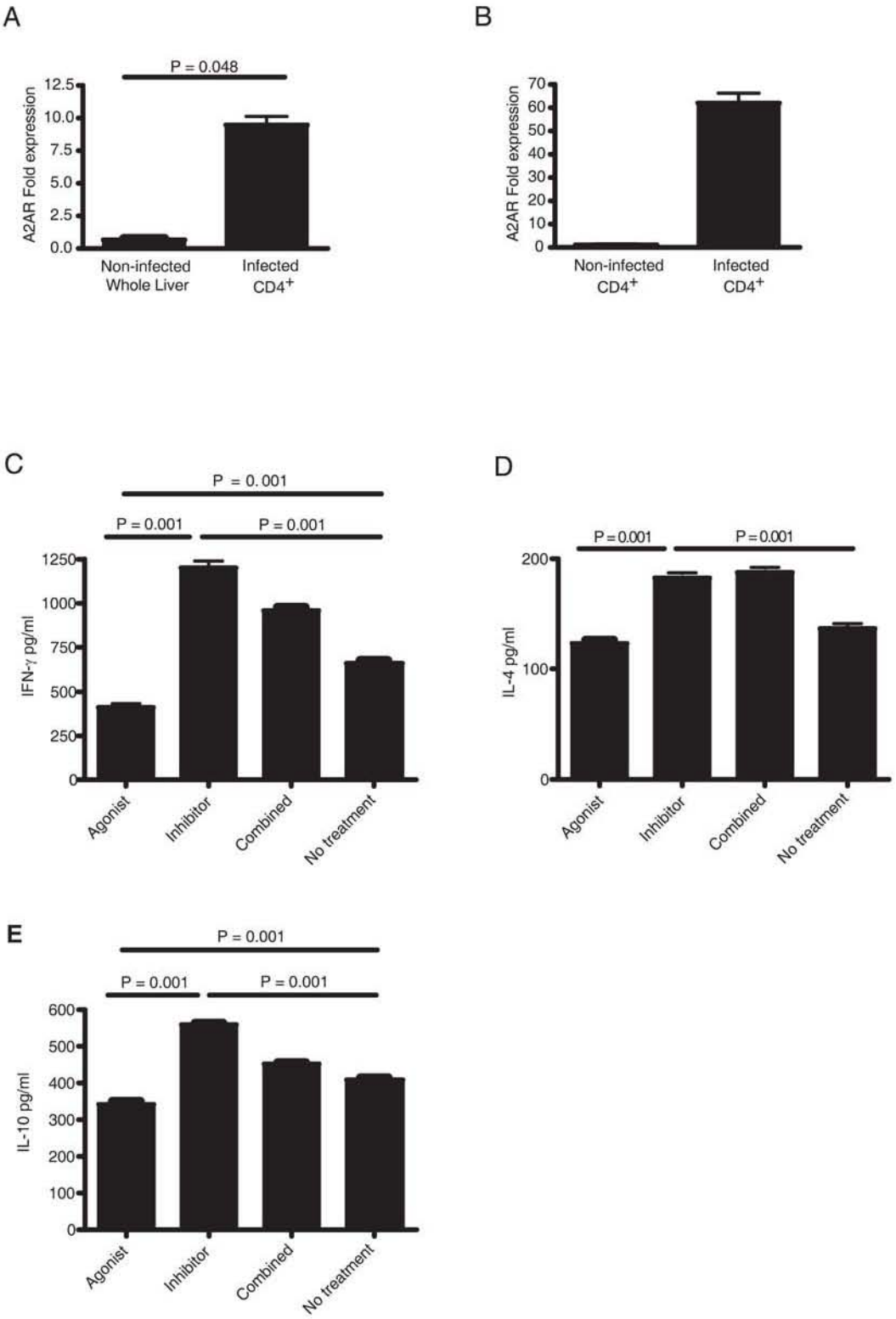
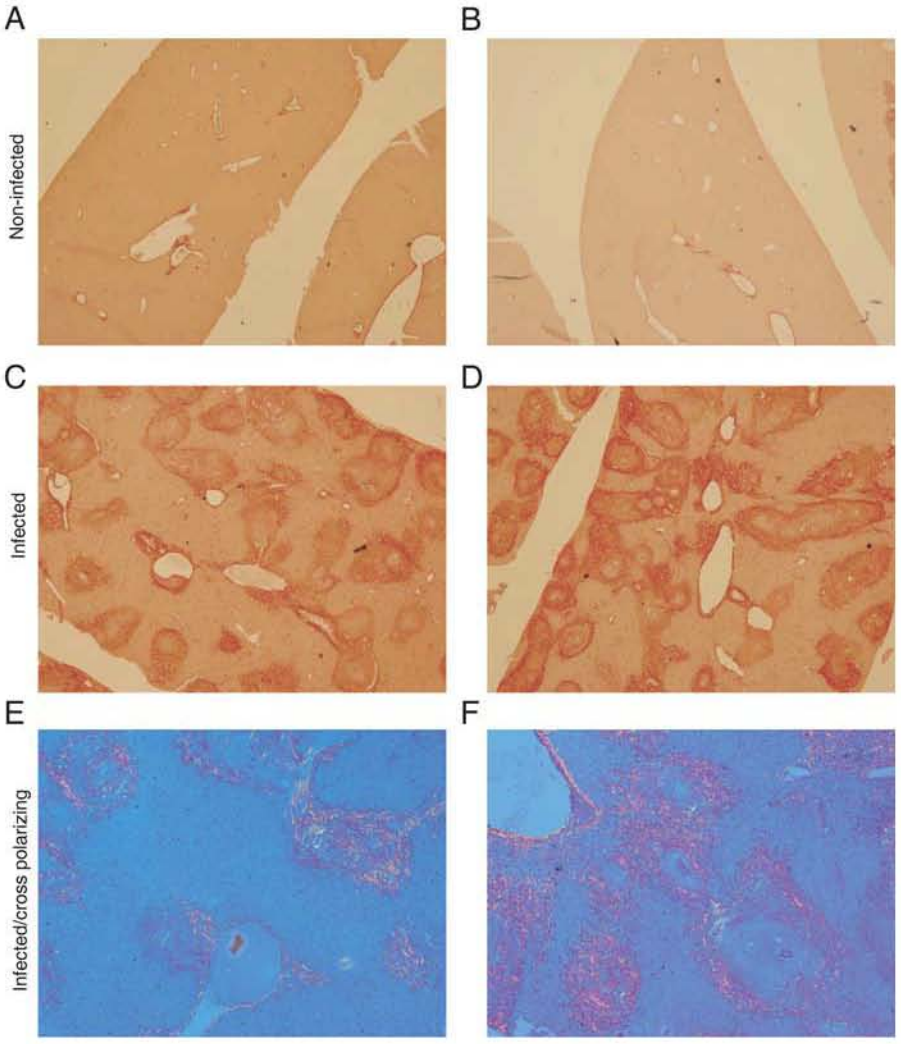

A
P = 0.048
A2AR Fold expression
12.5
10.0
7.5
5.0
2.5
0.0
Non-infected Whole Liver
Infected CD4+
B
A2AR Fold expression
70
60
50
40
30
20
10
0
Non-infected CD4+
Infected CD4+
C
P = 0.001
P = 0.001
P = 0.001
IFN-γ pg/ml
1250
1000
750
500
250
0
Agonist
Inhibitor
Combined
No treatment
D
P = 0.001
P = 0.001
IL-4 pg/ml
200
100
0
Agonist
Inhibitor
Combined
No treatment
E
P = 0.001
P = 0.001
P = 0.001
IL-10 pg/ml
600
500
400
300
200
100
0
Agonist
Inhibitor
Combined
No treatment

Figure 6: *S. mansoni* egg-induced pathology is increased by loss of A2A receptor signaling. Liver sections from WT mice (A,C,E) and $A2AR^{-/-}$ mice (B,D,F) were stained with picrosirius red. A and B are sections from non-infected mice. C and D are sections from mice that were infected for 8 weeks. E and F are sections from mice that were infected for 8 weeks and viewed under crossed polarizing filters. G) Quantitative levels of collagen from digital micrographs of stained liver sections from 8 weeks infected WT and $A2AR^{-/-}$ mice.


A
B
Non-infected
C
D
Infected
E
F
Infected/cross polarizing

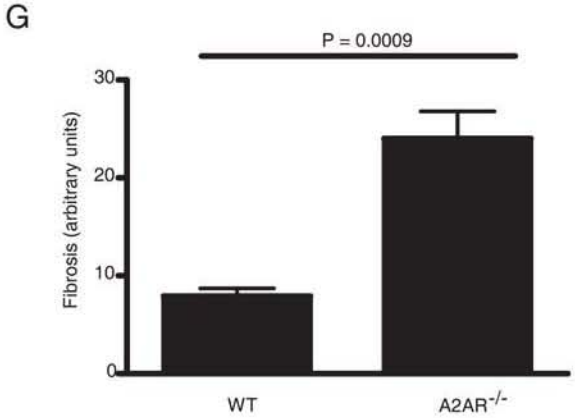

G
P = 0.0009
30
20
10
0
Fibrosis (arbitrary units)
WT
A2AR-/-

Figure 7: A2A receptor modulates Th2 responses during patent *S. mansoni* infection. A) Splenic $CD4^+$ T cells from 8 weeks infected WT and $A2AR^{-/-}$ mice were isolated by MACS separation columns and cytokine mRNA levels from extracted RNA was measured by Real-time PCR. Fold expression of IFN-γ, IL-4, IL-5, IL-10 and IL-13 cytokines was normalized to mRNA cytokine levels from infected WT mice. B) Splenocytes from 8 weeks infected WT and $A2AR^{-/-}$ mice were stimulated for 4 hours with anti-CD3 and then treated with BD GolgiStop for 2 hours. Cells were harvested, stained with anti-CD4 PercP Cy5.5, fixed, permeabilized and stained with FITC-conjugated anti-mouse IFN-γ, PE-conjugated anti-mouse IL-4 and APC-conjugated anti-mouse IL-10. Cells were analyzed using a LSR II Optical Bench flow cytometer. Numbers are percentages of total gated $CD4^+$ T cells and numbers in parenthesis represent the Mean Fluorescence Intensity (MFI) of those cells. C) Splenic $CD4^+$ T cells from 8 weeks infected WT and $A2AR^{-/-}$ mice were isolated by MACS separation columns and co-cultured with splenic dendritic cells from non-infected WT mice at a ratio of 1:10 (4×10^6 T cells: 400,000 DC's) and stimulated with SEA (■) or not stimulated (□) for 3 days. IFN-γ, IL-4, IL-5, IL-10 and IL-13 cytokine protein levels were measured by ELISA.

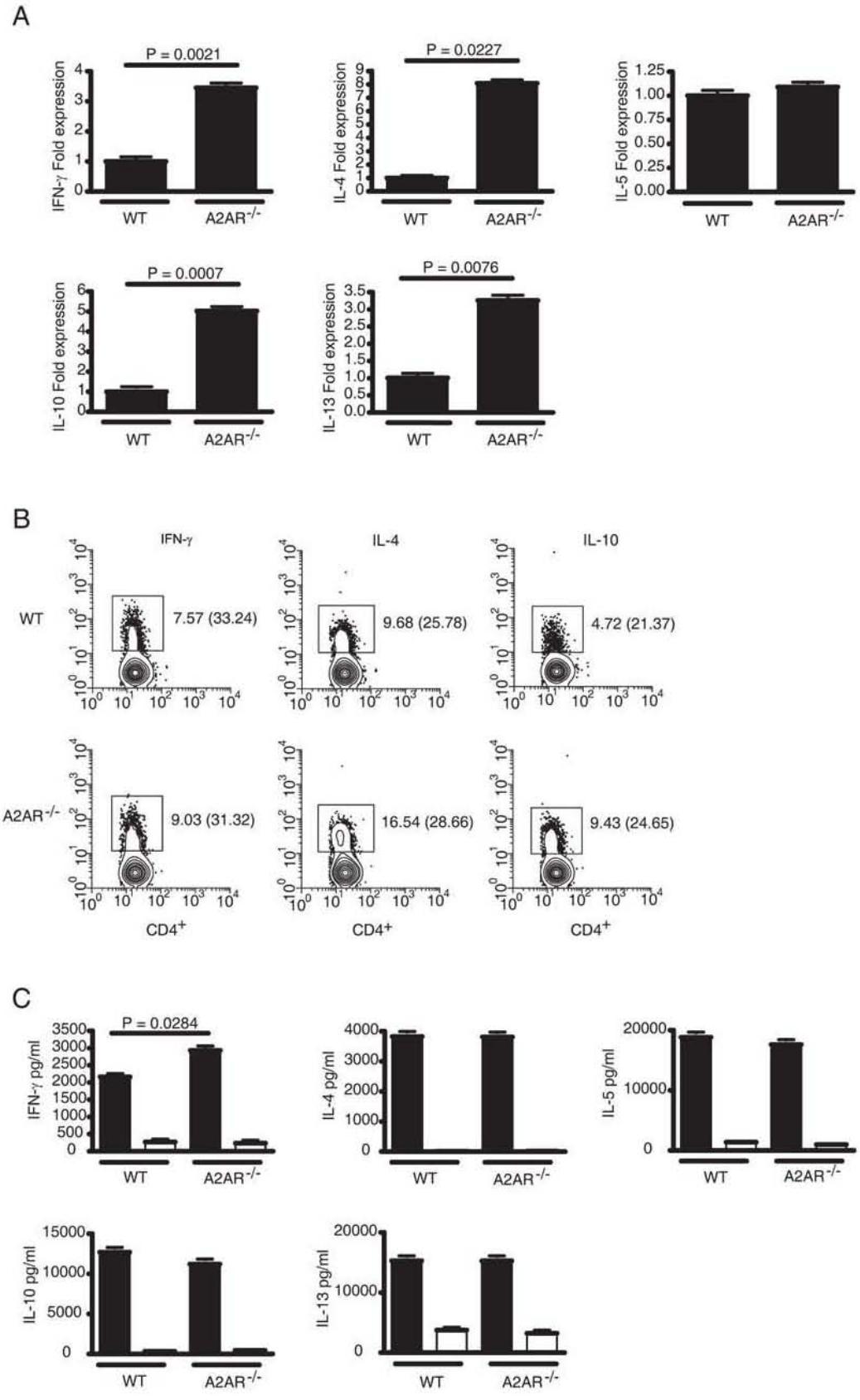
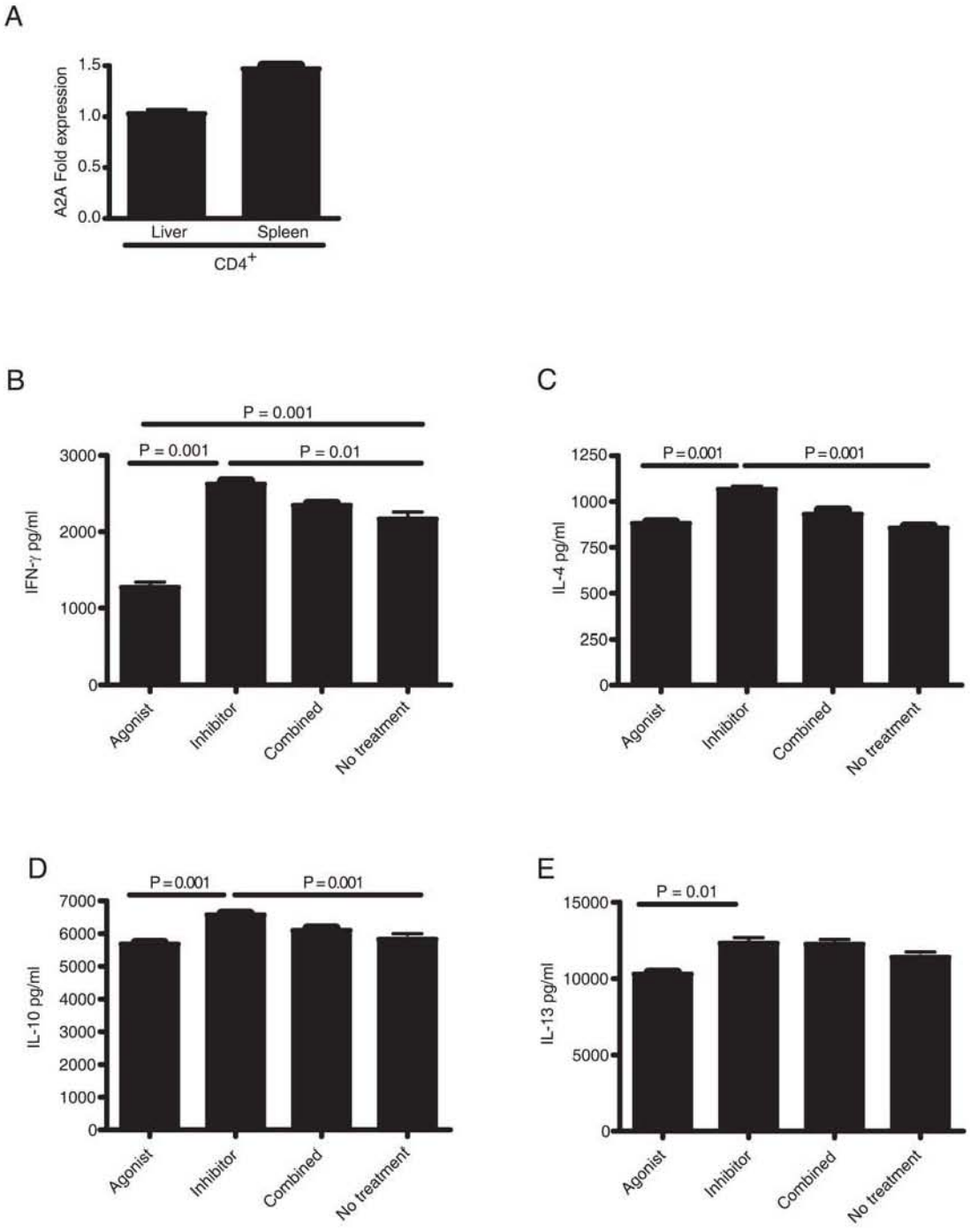

A
P = 0.0021
IFN-γ Fold expression
WT
A2AR-/-
P = 0.0227
IL-4 Fold expression
IL-5 Fold expression
P = 0.0007
IL-10 Fold expression
P = 0.0076
IL-13 Fold expression
B
IFN-γ
IL-4
IL-10
WT
7.57 (33.24)
9.68 (25.78)
4.72 (21.37)
A2AR-/-
9.03 (31.32)
16.54 (28.66)
9.43 (24.65)
CD4+
C
P = 0.0284
IFN-γ pg/ml
IL-4 pg/ml
IL-5 pg/ml
IL-10 pg/ml
IL-13 pg/ml

Figure 8: Direct regulation of $CD4^+$ T cell cytokine production by A2A receptor during Th2 responses. A) RNA from splenic $CD4^+$ T cells of 8 weeks infected WT mice were extracted and A2AR mRNA levels was measured by Real-time PCR. Fold expression of A2AR was normalized to A2AR mRNA levels from hepatic $CD4^+$ T cells of 4 weeks infected WT mice. Splenic $CD4^+$ T cells from 8 weeks infected C57 BL/6 WT mice were isolated and incubated for 18 hrs treated with either 1 uM A2AR Agonist (CGS), 1 uM A2AR Inhibitor (ZM), 1 uM Agonist + 1 uM Inhibitor, or not treated. Cells were then co-cultured with splenic dendritic cells from non-infected WT mice ($2x10^6$ T cells : 200,000 DC's) and stimulated with SEA for 3 days. B) IFN-γ, C) IL-4, D) IL-10 and E) IL-13 protein levels were measured by ELISA.


A
A2A Fold expression
1.5
1.0
0.5
0.0
Liver
Spleen
CD4+
B
P = 0.001
P = 0.001
P = 0.01
IFN-γ pg/ml
3000
2000
1000
0
Agonist
Inhibitor
Combined
No treatment
C
P = 0.001
P = 0.001
IL-4 pg/ml
1250
1000
750
500
250
0
Agonist
Inhibitor
Combined
No treatment
D
P = 0.001
P = 0.001
IL-10 pg/ml
7000
6000
5000
4000
3000
2000
1000
0
Agonist
Inhibitor
Combined
No treatment
E
P = 0.01
IL-13 pg/ml
15000
10000
5000
0
Agonist
Inhibitor
Combined
No treatment

Chapter 2

Role of primed schistosome-specific $CD4^+$ T cells on the growth and development of *Schistosoma mansoni* worms in early infection

Introduction

Exploitation of the host by parasites is a well-recognized but poorly understood phenomenon. The co-existence of intricate host immune responses and parasite immune evasion mechanisms in host-parasite relationships is widely regarded as evidence of extensive co-evolution of parasites and their hosts over extended periods of evolutionary time. Indeed, mechanisms that allow parasites to evade vigorous host immune responses which would otherwise result in parasite elimination are a hallmark of parasite survival. Further complexity is evident in some parasite-host relationships, where parasites actively co-opt the host immune system for their own survival. This is the case with parasitic trematodes of the genus *Schistosoma,* also known as blood flukes. Previous studies have shown that the host's adaptive immune system is needed for *S. mansoni* growth and development (Amiri et al., 1992). Furthermore, it was shown in a murine model of *S. mansoni* infection that $CD4^+$ T cells were critical for allowing parasite growth and development to proceed, since reconstitution of Recombination Activating Gene deficient ($RAG^{-/-}$) mice, which lack all T and B lymphocytes, with $CD4^+$ T cells alone was sufficient to restore normal parasite development and egg production (Davies et al., 2001).

Paradoxically, several lines of evidence from human patients (Karanja et al., 2002) and laboratory animal studies (Hewitson et al., 2005) indicate that $CD4^+$ T cell responses to parasite antigens can also mediate significant levels of protection against schistosome infection. In animal models, this has been repeatedly shown in vaccination studies whereby *S. mansoni* cerceriae, the infective stage of the parasite, are irradiated to disrupt them by impairing the neuromuscular function in developing schistosomula and

slowing their migration through the host (Harrop and Wilson, 1993). The slow migration leads to a vigorous IFN-γ (Smythies et al., 1992) response by $CD4^+$ T (Vignali et al., 1989) cells that is protective against a subsequent infection with normal *S. mansoni* cerceriae. This protection reaches its highest level after 4-6 weeks post-infection with irradiated cerceriae, leading to a decrease of ~50-60% in worm burdens after challenge with normal cerceriae (Hewitson et al., 2005).

These seemingly contradictory observations, that $CD4^+$ T cells can both promote and inhibit schistosome infection, raise the important question of whether these properties of $CD4^+$ T cells are linked and occur simultaneously, or whether inhibitory and growth-promoting effects are independent or even mutually antagonistic. In an attempt to answer this question, we assessed the ability of effector $CD4^+$ T cells, primed to respond to schistosome antigens by infection or vaccination, to enhance the growth and development of *S. mansoni* worms when compared to naïve $CD4^+$ T cells. We evaluated the role of effector $CD4^+$ T cells that recognize and mount responses to foreign peptide antigens presented in the context of major histocompatibility complex (MHC) class II molecules, by examining schistosome development in an *in vivo* context where schistosome- specific effector $CD4^+$ T cells are enriched and present in higher numbers. Through the evaluation of *S. mansoni* growth and development by adoptive transfer of primed $CD4^+$ T cells into $RAG^{-/-}$ mice, we demonstrate that these cells restore *S. mansoni* development to the same extent as naïve $CD4^+$ T cells. Our data therefore provide evidence that $CD4^+$ T cell responses to schistosome worm antigens do not interfere with the growth- and development-promoting abilities of $CD4^+$ T cells. Further, these data suggest that the presence of primed, schistosome-specific $CD4^+$ T cells does not facilitate schistosome

development to a greater extent that when only naïve cells are present. Finally, we show that even in vaccinated immunocompetent mice where approximately 50 % protection is induced, the development of the challenge parasites that survive immune elimination proceeds normally. Taken together, our data suggest that the facilitation of schistosome development by $CD4^+$ T cells is unaffected by specific responses to schistosome antigens by these cells and that, paradoxically, $CD4^+$ T cells can both facilitate schistosome development and simultaneously mediate protection against schistosome infection.

Materials and Methods

Mice

Wild-type C57BL/6 mice were purchased from National Cancer Institute (NCI), (Frederick, MD). Breeding pairs of RAG-$1^{-/-}$ mice on a C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME) and bred in-house to generate sufficient numbers for experiments. All studies involving animals were performed in accordance with protocols approved by the relevant Institutional Animal Care and Use Committees.

Schistosome infection and vaccination

Cercariae of *Schistosoma mansoni* (Puerto Rican strain) were obtained from infected *Biomphalaria glabrata* snails. Mice were infected by immersion of the tail for 40 min in water containing 150 *S. mansoni* cercariae. Mice were sacrificed at 6 weeks post infection. For experiments where animals were vaccinated prior to infection, mice were first exposed to 500 *S. mansoni* cercariae irradiated with 50 krad from a ^{60}Co source. Mice were then infected 4 weeks later as described and euthanized at 6 weeks post infection. In all experiments, groups of wild-type and RAG-$1^{-/-}$ mice were exposed at the same time to parasites from the same cercarial pool.

Parasite recovery and measurement of parasitological parameters

Parasites were recovered from the portal system by perfusion, immediately fixed in 4% neutral-buffered formaldehyde and photographed using a Nikon Coolpix 4500 4.0 megapixel digital camera connected to a Vistavision trinocular dissecting microscope at 20× magnification. Length of male parasites was determined from digital images using ImageJ software (http://rsb.info.nih.gov/ij). Quantitative analysis of parasite length was performed on male worms as male schistosomes always outnumber females in experimental infections and female growth is significantly influenced by pairing with males (Hernandez et al., 2004). Liver tissue was digested in 0.7% trypsin (50 ml) in PBS for 2–3 h at 37 °C and eggs were counted under a dissecting microscope.

Parasite antigen preparation

For preparation of soluble worm antigen (SWAP), adult *S. mansoni* worms (male and female) were suspended in ice-cold PBS and homogenized on ice. Insoluble material was removed by centrifugation at 13,200 x g for 45 min at 4°C, and the supernatant was filter-sterilized. Protein concentration was determined by the Bradford assay (BioRad, Hercules, CA) on a Spectramax M2 micro plate spectrophotometer (Molecular Devices, Sunnyvale, CA) and frozen in aliquots stored –80°C.

$CD4^+$ T cell isolation and Cell culture

Single-cell suspensions of leukocytes were prepared from pooled spleens or pooled livers of infected mice by dissociating tissues with wash medium through nylon cell strainers and lysing erythrocytes with ACK lysing buffer if necessary. For livers, 25 ml of 35% Percoll (GE Healthcare Bio-Sciences AB) was added to each liver single-cell suspension and centrifuged at 2000 rpm for 10 min at room RT to isolate the leukocyte pellet from the raft of hepatocytes. $CD4^+$ T cells were isolated by positive selection using magnetic anti-CD4 microbeads and MACS separation columns (Miltenyi Biotech, CA) and following the instructions provided by the manufacturers. $CD4^+$ cells were cultured in T cell medium (TCM; RPMI 1640, 10% heat-inactivated FCS, 1000 U/ml penicillin, 10 μg/ml streptomycin, HEPES, 10mM; MEM Non-essential amino acids Solution, 10mM; L-glutamine, 200mM; Sodium pyruvate, 100mM; β-mercaptoethanol, 55mM) in the presence of $CD11c^+$ splenic dendritic cells (1:10 ratio of $CD4^+$ T cell:dendritic cell) isolated from non-infected C57BL/6 mouse spleens using magnetic anti-$CD11c^+$microbeads and MACS separation columns. SWAP was added to a final concentration of 50μg/ml. Positive and negative control cultures received 1μg/ml anti-CD3 antibody or no antigen, respectively. Following incubation for 72 h at 37°C in 5% carbon dioxide, supernatants were collected and immediately frozen at –80°C for subsequent determination of cytokine concentration.

Reconstitution of RAG-$1^{-/-}$ mice with wild-type $CD4^{+}$ lymphocytes

Pooled spleens or livers from wild-type C57BL/6 mice were dispersed through a 70-μm nylon strainer. Cells were incubated with anti-CD4 coated microbeads (Miltenyi Biosciences) and separated using Midi-Macs magnetic columns (Miltenyi Biosciences). Four times 10^6 cells (0.5x10^6, 0.8x10^6 for livers) were transferred into RAG-$1^{-/-}$ mice by i.v. injection into a lateral tail vein. Recipient animals were then infected with cercariae 24 h later, as described above. To verify the efficacy of adoptive transfers at necropsy, splenocytes from reconstituted RAG-$1^{-/-}$ mice were surface labelled with allophycocyanin (APC)-Cy7-conjugated antibodies to CD4, fluorescein isothiocyanate (FITC)-conjugated antibodies to CD8, phycoerythrin (PE)-conjugated antibodies to NK1.1 and PerCp-Cy5.5-conjugated antibodies to CD19 (BD Biosciences) and analysed using a LSR II Optical Bench flow cytometer with FACSDiva and Winlist software, version 5.0 (Verity Software House).

Cytokine analysis

The concentrations of cytokines in culture supernatants and plasma were determined using sandwich ELISA kits from BD Biosciences/Pharmingen (San Diego, CA), following the instructions issued by the manufacturer. Briefly, for each cytokine, flat-bottomed plates (Immulon 2HB; Thermo, MA) were each coated overnight at 4 °C with the appropriate capture antibody diluted in carbonate-bicarbonate coating buffer (pH 9.5). Following an initial wash with PBS (pH 7.2) containing 0.05% Tween 20 (PBST; Sigma),

the plates were blocked with 10% fetal calf serum (FCS) in PBS for 1 h at room temperature. Samples or standards were then added to the wells and the plates were incubated for 2 hours at RT. After incubation, the plates were washed and 100 µl of working detector containing biotinylated anti-mouse cytokine antibody and Streptavidin–HRP (horseradish peroxidase conjugate) was added in a one-step incubation for 1 hour. After 7-10 washes, the substrate solution TMB and hydrogen peroxide was added to each well and 30 minutes after incubation in the dark, the reaction was stopped with 2N H_2SO_4 Absorbance was read at 450 nm using a Spectramax M2 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) and corrected for plate absorbance at 570 nm. Concentrations of cytokines (pg/ml) in the samples were determined by interpolation from standard curves calculated for each plate using appropriate amounts of recombinant mouse cytokines.

Statistical analysis

Because unequal variances were observed among some of the groups analysed in this study, stringent non-parametric tests were used throughout to test the significance of differences between experimental groups. For two groups, significance of differences between experimental groups was tested using Mann–Whitney tests, and for three groups the significance of differences was tested using Kruskal–Wallis tests followed by Dunns' multiple comparison tests. Statistical analyses were performed with GraphPad Prism Version 4.0 software (GraphPad Software, Inc., San Diego, CA). *P* values of less than 0.05 were considered significant. All experiments were performed twice with similar results.

Results

Splenic $CD4^+$ T cells primed to schistosome antigens restores schistosome development but does not confer protection in $RAG^{-/-}$ mice

To test whether $CD4^+$ T cells that have previously encountered schistosome antigens would restore schistosome development to the same extent as naïve cells, splenic $CD4^+$ T cells were isolated from wild type mice at 4 weeks post-infection with *S. mansoni* cerceriae and adoptively transferred into $RAG^{-/-}$ recipient mice. Before adoptive transfer, splenic $CD4^+$ T cells from 4 week -infected wild type mice were re-stimulated *in vitro* with dendritic cells and SWAP to determine if they were primed to respond to schistosome antigens. Upon stimulation with SWAP, primed $CD4^+$ T cells produced high levels of IFN-γ (Fig. 11A) compared to naïve $CD4^+$ T cells from wild type mice that were not infected, demonstrating that cells from infected mice were indeed primed to respond to schistosome antigens. However, the length of parasites recovered 6 weeks later from $RAG^{-/-}$ mice that received primed $CD4^+$ T cells was the same as parasites recovered from $RAG^{-/-}$ mice that received naïve $CD4^+$ T cells (Fig. 11B). Egg production per worm pair, which indicates the fecundity of the parasite, was also not significantly different between the two groups (Fig. 11C). These results indicate that $CD4^+$ T cells primed to respond to schistosome antigens restored worm development to the same extent as naïve cells.

To determine if primed $CD4^+$ T cells played any role in protection against *S. mansoni* infection, we assessed the number of worms recovered from the two groups of recipient $RAG^{-/-}$ mice. The number of worms recovered from $RAG^{-/-}$ mice that received primed $CD4^+$ T cells was not significantly different from the number recovered from recipients

of naïve $CD4^+$ T cells (Fig. 11D), showing that primed $CD4^+$ T cells do not confer protection to schistosomes in early infection. Indeed, we were able to recover comparable numbers of worms from $RAG^{-/-}$ recipient mice that did not receive any cells, indicating that, in primary infections, $CD4^+$ T cells do not protect against schistosome infection, while simultaneously facilitating schistosome development.

Priming of hepatic $CD4^+$ T cells by schistosome infection does not affect their ability to promote *S. mansoni* growth and development

Unlike the spleen, which is a secondary lymphoid organ distal to the actual site of infection, the liver is the anatomical site at which developing *S. mansoni* worms and their antigens concentrate and the liver therefore contains $CD4^+$ T cells that are actively responding to schistosome infection (Makarem and Davies, submitted). We therefore hypothesized that antigen priming of $CD4^+$ T cells in the liver may have a more pronounced effect on the ability of these cells to influence worm development than priming at other anatomic sites. To test this hypothesis, $CD4^+$ T cells from the livers of either 4 week-infected or non-infected wild type mice were isolated and adoptively transferred to $RAG^{-/-}$ recipient mice. Six weeks after infecting the $RAG^{-/-}$ recipient mice, parasites recovered from $RAG^{-/-}$ mice that received primed hepatic $CD4^+$ T cells were the same length as parasites recovered from $RAG^{-/-}$ mice that received naïve hepatic $CD4^+$ T cells (Fig. 12A). Egg production per worm pair was also similar between both groups (Fig. 12B). Thus, antigen priming of hepatic $CD4^+$ T cells does not affect the ability of these cells to promote schistosome development. Further, as with splenic $CD4^+$ T cells

(Fig. 11D), the number of worms recovered from $RAG^{-/-}$ mice that received either primed or naïve hepatic $CD4^+$ T cells was comparable (Fig. 12C), again indicating that neither naïve nor primed $CD4^+$ T cells of hepatic origin confer protection to schistosome infection.

Splenic $CD4^+$ T cells from vaccinated mice restore *S. mansoni* development when transferred to $RAG^{-/-}$ mice

Vaccination by exposure to radiation-attenuated cercariae induces a vigorous Th1 response that is protective against subsequent challenge with non-attenuated *S. mansoni* cercariae (Hewitson et al., 2005). To assess whether priming of $CD4^+$ T cells by vaccination would alter the ability of these cells to facilitate worm development, splenic $CD4^+$ T cells from either vaccinated or naive wild type mice were adoptively transferred into $RAG^{-/-}$ recipient mice. $RAG^{-/-}$ recipients were then infected and parasite development assessed six weeks later. Parasites recovered from $RAG^{-/-}$ mice that received $CD4^+$ T cells from vaccinated animals were similar in length to worms recovered from $RAG^{-/-}$ mice that received naïve $CD4^+$ T cells (Fig. 13A). This suggests that vaccine-induced $CD4^+$ T cell responses to *S. mansoni* worm antigen do not interfere with the ability of $CD4^+$ T cells to facilitate schistosome development. Interestingly, the numbers of parasites recovered from $RAG^{-/-}$ mice that received $CD4^+$ T cells from vaccinated donors were not significantly different from those recovered from $RAG^{-/-}$ mice that received naïve $CD4^+$ T cells (Fig. 13B). Indeed, and contrary to expectations, we recovered more worms from $RAG^{-/-}$ mice that received $CD4^+$ T cells from vaccinated animals, showing that the ability of $CD4^+$ T cells to mediate protection is lost upon adoptive transfer.

Vaccination confers protection against subsequent challenge, but has no effect on *S. mansoni* worm growth and development

Exposure to irradiated cercariae has been shown to induce a strong Th1 response (IFN-γ) that mediates approximately 50 % protection to subsequent challenge with infectious cercariae (James et al., 1987). Our data in Fig. 13 suggest this protective response does not interfere with the ability of $CD4^+$ T cells to facilitate schistosome development. However, because adoptive transfer of $CD4^+$ T cells from vaccinated mice into $RAG^{-/-}$ recipients also appeared to abrogate the protective properties of these cells, we examined schistosome development directly in wild type mice that were partially protected by prior vaccination. Wild type mice exposed to irradiated cercariae were challenged 4 weeks later with normal live cercariae. Then, six weeks after challenge, worm burden and parasite development was compared to that in non-vaccinated mice. Consistent with previous reports (James et al., 1987), worm numbers (Fig. 14A) in vaccinated mice were ~50% lower than those in unvaccinated mice. However, the parasites recovered from the vaccinated animals were the same length (Fig. 14B) and produced similar numbers of eggs per worm pair (Fig. 14C) to those isolated from non-vaccinated controls. These data suggest that $CD4^+$ T cell responses induced by vaccination do not interfere with the development of those parasites that evade vaccine-induced immunity.

Discussion

$CD4^+$ T cells play a significant role in the growth of *Schistosoma* worms and their development into mature, reproductively active adult parasites (Lamb et al., 2007). However, previous studies have not examined the mechanisms by which $CD4^+$ T cells mediate these effects on blood fluke development. Because the primary function of $CD4^+$ T cells is to recognize and respond to foreign peptide antigens of extracellular origin that are presented in the context of MHC class II molecules, we hypothesized that $CD4^+$ T cell responses to schistosome antigens during early infection would be implicated in the remarkable ability of $CD4^+$ T cells to influence schistosome development. We therefore evaluated whether $CD4^+$ T cells that were previously primed to respond to *S. mansoni* worm antigens, specifically in early infection and before the response to egg antigens, would have altered abilities to facilitate worm development. Here we show that $CD4^+$ T cells from the spleen, primed to *S. mansoni* worm antigens in early infection, restore worm development and egg production to the same level as naïve $CD4^+$ T cells that are not antigen-experienced (Fig. 11). Additionally, primed $CD4^+$ T cells from the liver, where the $CD4^+$ T cell response is more pronounced than in the spleen, did not enhance worm development or egg production beyond the levels obtained with naïve cells (Fig. 12). Taken together, these results suggest that the $CD4^+$ T cell responses to schistosome worm antigens elicited during primary infection do not positively affect *S. mansoni* growth and development

We next tested whether $CD4^+$ T cells from vaccinated animals, which mount a pronounced and protective response to *S. mansoni* worm antigens, would exhibit altered abilities to enhance *S. mansoni* development. Interestingly, adoptive transfer of these

cells into $RAG^{-/-}$ mice restored worm development to the same extent as naïve $CD4^+$ T cells (Fig. 13), again suggesting that effector responses by $CD4^+$ T cells do not affect the development of *S. mansoni* worms. An alternative explanation for our results is that homeostatic proliferation in the $RAG^{-/-}$ recipients could cause the primed $CD4^+$ T cells to now become unresponsive and worm development was therefore restored to levels indistinguishable to those seen with naïve cells. Contrary to our expectations, we have preliminary evidence suggesting that while effector $CD4^+$ T cells continue to be responsive to worm antigens after homeostatic proliferation, naïve $CD4^+$ T cells have also acquired that same phenotype (data not shown), further complicating the comparison between primed and naïve cells using this approach. Indeed, other studies have also shown that naïve $CD8^+$ T cells undergoing homeostasis-driven proliferation convert to a phenotype similar to that of memory T cells and acquire the ability to rapidly secrete IFN-γ and become cytotoxic effectors when stimulated with cognate antigen (Goldrath et al., 2000). This perhaps could explain why the effect of $CD4^+$ T cell responses on *S. mansoni* growth and development could not be assessed, since naïve $CD4^+$ T cells have also acquired a similar phenotype after adoptive transfer to $RAG^{-/-}$ mice.

Through adoptive transfer experiments using primed $CD4^+$ T cells, we also found that the presence of $CD4^+$ T cells primed by prior exposure to primary infection did not adversely affect the ability of schistosome larvae to establish infection in $RAG^{-/-}$ recipients, as similar numbers of parasites were recovered from recipients of naïve and primed cells (Fig. 13). Thus, while primed $CD4^+$ T cells do not enhance *S. mansoni* worm development, we also can find no evidence that $CD4^+$ T cell responses induced by primary infection can mediate protection against schistosomes. Indeed, $CD4^+$ T cells

from vaccinated animals, which can mediate protection against a subsequent challenge infection, also failed to mediate any protective effect after adoptive transfer to $RAG^{-/-}$ mice. This might suggest that there are other important factors, in addition to $CD4^+$ T cells, that play a role in mediating protection in vaccinated mice. Indeed, it was shown that optimal immunization against *S. mansoni* requires the simultaneous induction of both humoral and cell-mediated mechanisms (Jankovic et al., 1999), since B cell knockout mice displayed reduced protection against challenge infection, despite developing a normal IFN-γ response. Since $RAG^{-/-}$ mice lack a functional B cell compartment, this major factor could account for the failure to transfer protection in these strains.

Finally, we propose that $CD4^+$ T cells could simultaneously facilitate and antagonize schistosome development in vaccinated mice (Fig. 14). This result suggests that the protection, by $CD4^+$ T cells as well as other immune mechanisms, is occurring at the very early stage in infection and that those worms that do escape this protection are developing normally by the help of $CD4^+$ T cells (Vignali et al., 1989).

In conclusion, we show that schistosome development is not enhanced by $CD4^+$ T cell responses to worm antigens. Importantly, we show that $CD4^+$ T cell responses restore normal *S. mansoni* growth and development while also playing a role in protection against schistosome infections. This suggests that $CD4^+$ T cells have a dual function in both facilitating schistosome worm development while simultaneously protecting against infection.

Figure 9: Adoptive transfer of splenic $CD4^+$ T cells into RAG-$1^{-/-}$ mice from previously infected donors restore *S. mansoni* growth and egg production, but does not confer protection against *S. mansoni* infection. (A) $CD4^+$ T cells were isolated from spleens of 4 week-infected (Primed) or non-infected (Naïve) wild type mice, cultured ($2x10^6$ cells/ml) with dendritic cells and stimulated with SWAP antigen (■) or not stimulated (□). IFN-γ cytokine concentrations in the culture supernatants were measured by ELISA 72 h after stimulation. (B-D) Splenic $CD4^+$ T cells from wild type donors that were either previously infected with *S. mansoni* cercariae for 4 weeks (Primed) or not infected (Naïve), were adoptively transferred into RAG-$1^{-/-}$ mice (4×10^6 cells/recipient). The $RAG^{-/-}$ recipients were then infected with *S. mansoni* cercariae and worm length (B), egg production per worm pair (C), and the number of worms (D) per mouse was assessed after 6 weeks.

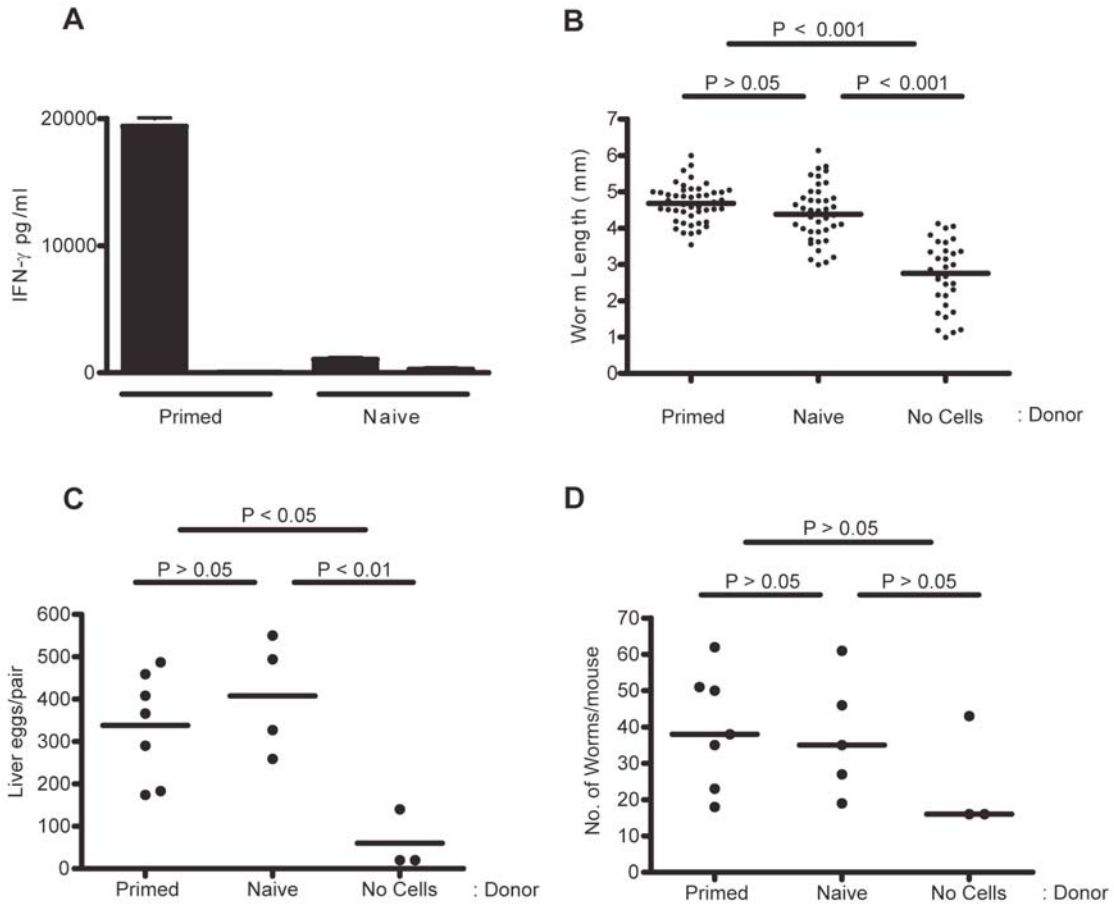

A
IFN-γ pg/ml
20000
10000
0
Primed
Naive
B
P < 0.001
P > 0.05
P < 0.001
Worm Length (mm)
7
6
5
4
3
2
1
0
Primed
Naive
No Cells
: Donor
C
P < 0.05
P > 0.05
P < 0.01
Liver eggs/pair
600
500
400
300
200
100
0
Primed
Naive
No Cells
: Donor
D
P > 0.05
P > 0.05
P > 0.05
No. of Worms/mouse
70
60
50
40
30
20
10
0
Primed
Naive
No Cells
: Donor

Figure 10: Hepatic $CD4^+$ T cells from previously infected donors restore *S. mansoni* growth, but do not confer protection against *S. mansoni* infection when adoptively transferred into RAG-$1^{-/-}$ mice. Hepatic $CD4^+$ T cells from wild type donors that were either previously infected with *S. mansoni* cercariae for 4 weeks (Primed) or not infected (Naïve), were adoptively transferred into RAG-$1^{-/-}$ mice (0.8×10^6 cells/recipient). The $RAG^{-/-}$ recipients were then infected with *S. mansoni* cercariae and assessed after 6 weeks for worm length (A), egg production per worm pair (B) and number of worms per mouse (C)

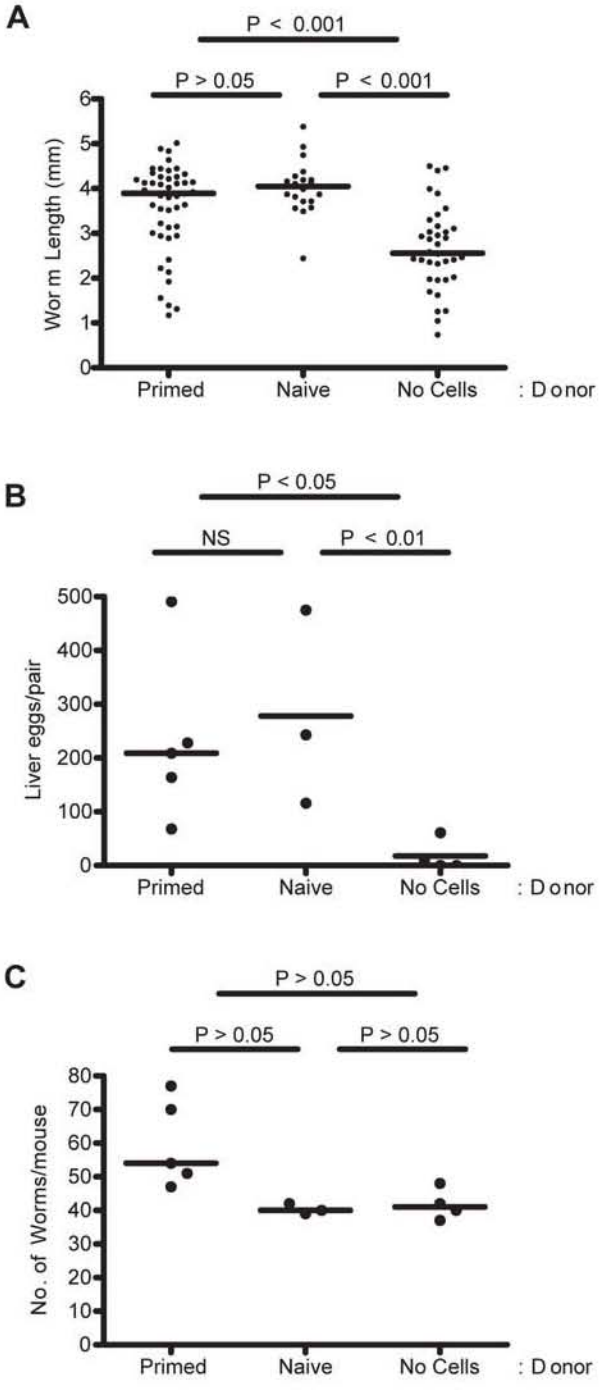

A
P < 0.001
P > 0.05
P < 0.001
Worm Length (mm)
0
1
2
3
4
5
6
Primed
Naive
No Cells
: Donor
B
P < 0.05
NS
P < 0.01
Liver eggs/pair
0
100
200
300
400
500
Primed
Naive
No Cells
: Donor
C
P > 0.05
P > 0.05
P > 0.05
No. of Worms/mouse
0
10
20
30
40
50
60
70
80
Primed
Naive
No Cells
: Donor

Figure 11: Adoptive transfer of Splenic $CD4^+$ T cells into RAG-$1^{-/-}$ mice from previously vaccinated donors restore the development of *S. mansoni* worms, but does not confer protection against *S. mansoni* infection. Splenic $CD4^+$ T cells from wild type donors that were either previously vaccinated with irradiated *S. mansoni* cercariae for 4 weeks or not infected (Naïve), were adoptively transferred into RAG-$1^{-/-}$ mice (4×10^6 cells/recipient). The $RAG^{-/-}$ recipients were then infected with *S. mansoni* cercariae and worm length (A) and number of worms (B)) assessed after 6 weeks.

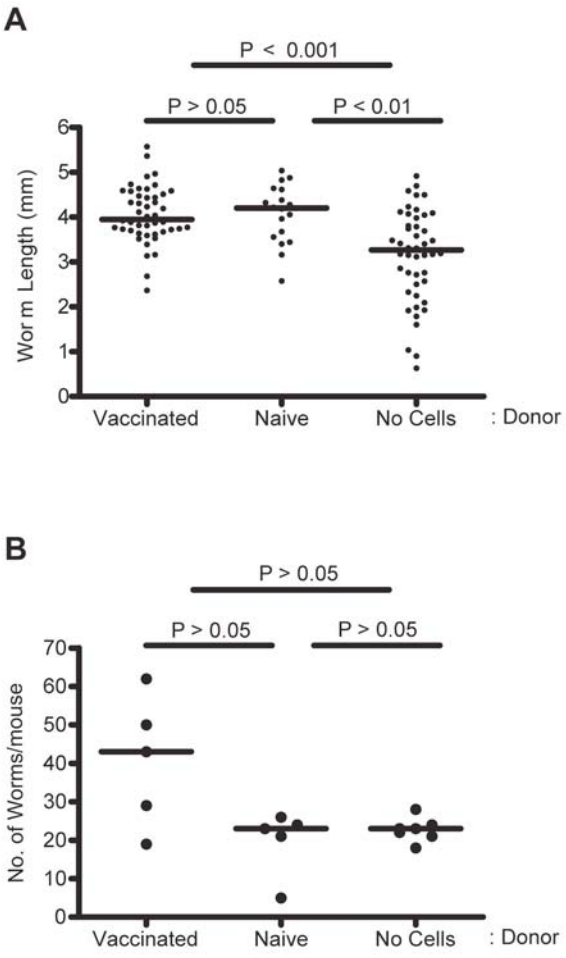
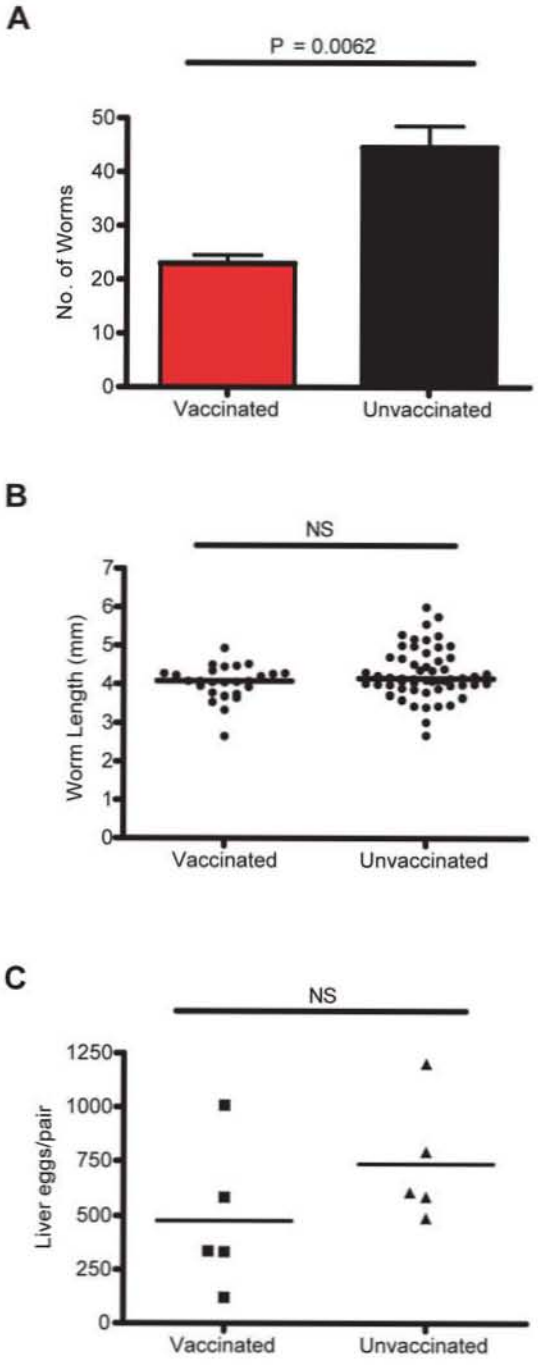

A
P < 0.001
P > 0.05
P < 0.01
Wor m Length (mm)
0
1
2
3
4
5
6
Vaccinated
Naive
No Cells
: Donor
B
P > 0.05
P > 0.05
P > 0.05
No. of Worms/mouse
0
10
20
30
40
50
60
70
Vaccinated
Naive
No Cells
: Donor

Figure 12: Vaccination confers protection against the establishment of *S. mansoni* worms, but does not interfere with *S. mansoni* development. Mice were vaccinated with irradiated cercariae and then challenged with live cercariae 4 weeks later. After another 6 weeks, worm burden (A), worm length (B), and egg production per worm pair (C) was assessed and compared to parasites obtained from animals that were infected but not vaccinated.


A
P = 0.0062
No. of Worms
50
40
30
20
10
0
Vaccinated
Unvaccinated
B
NS
Worm Length (mm)
7
6
5
4
3
2
1
0
Vaccinated
Unvaccinated
C
NS
Liver eggs/pair
1250
1000
750
500
250
0
Vaccinated
Unvaccinated

Chapter 3

Role of IL-10 and TGF-β in the establishment and development of *Schistosoma mansoni* worms

Introduction

It is estimated that two billion people, or approximately one third of the world's population, suffer from helminth (worm) infections (Colley et al., 2001). Schistosomiasis, a helminth infection caused by trematode parasites of the genus *schistosoma*, is among the most important helminth infections, infecting approximately 200 million people worldwide and accounting for roughly one tenth of all helminth infections (Organization, 1993). Similar to other parasites, schistosomes have evolved mechanisms to evade protective host immune responses for their continual survival. Understanding the mechanisms that lie behind these evasive strategies might identify ways to disrupt them and could lead to the development of novel cures for this disease.

Our recent data has shown that IL-10 production by $CD4^+$ T cells is stimulated early during *S. mansoni* infection, suggesting that perhaps immune regulatory mechanisms induced in early infection allow *S.mansoni* worms to evade the immune response and become established. Furthermore, published data suggest that IL-10 interferes with vaccine-induced immunity to schistosomes, as a decrease in worm burden is observed in vaccinated IL-$10^{-/-}$ mice (Hoffmann et al., 1999). However in primary infections, lack of IL-10 does not seem to interfere with the establishment of schistosome worms (Wynn et al., 1998), suggesting that other regulatory factors might be compensating for the lack of that IL-10.

One such factor is the surface-bound or secreted cytokine Transforming Growth Factor-β (TGF–β). The TGF-β superfamily of polypeptide growth factors plays a prominent role in cell growth and differentiation across a wide range of species, from nematode to human. TGF-β plays a role in suppressing the differentiation, survival, and

proliferation of T cells (Rubtsov and Rudensky, 2007). For example, in a Trichenella spiralis model of infection, neutralizing TGF-β in IL-$10^{-/-}$ mice resulted in a decrease of larva establishment in muscle tissue (Beiting et al., 2007), suggesting that regulatory responses are induced as an evasive strategy to avoid the host protective immune response. In addition, the TGF-β-like receptor/Smad signaling pathway in parasitic helminths themselves has been shown to be important in regulating schistosome development (Beall et al., 2000).

In this study we assessed whether disrupting both IL-10 and TGF-β responses in a schistosome infection had a negative effect on *S. mansoni* worm establishment and development. Here, we provide evidence showing that although absence of IL-10 and TGF-β do not significantly affect the establishment of *S. mansoni* worms, normal development of these worms is affected.

Materials and Methods

Mice

Wild-type C57BL/6 mice were purchased from National Cancer Institute (NCI), (Frederick, MD). Six-wk-old C57BL/6 IL-$10^{-/-}$ mice were purchased from Jackson. All studies involving animals were performed in accordance with protocols approved by the relevant Institutional Animal Care and Use Committees.

Schistosome infection

Cercariae of *Schistosoma mansoni* (Puerto Rican strain) were obtained from infected *Biomphalaria glabrata* snails. Mice were infected by immersion of the tail for 40 min in water containing 150 *S. mansoni* cercariae. Mice were sacrificed at 6 weeks post infection. In all experiments, groups of wild-type and IL-$10^{-/-}$ mice were exposed at the same time to parasites from the same cercarial pool.

Parasite recovery and measurement of parasitological parameters

Parasites were recovered from the portal system by perfusion, immediately fixed in 4% neutral-buffered formaldehyde and photographed using a Nikon Coolpix 4500 4.0 megapixel digital camera connected to a Vistavision trinocular dissecting microscope at

20× magnification. Length of male parasites was determined from digital images using ImageJ software (http://rsb.info.nih.gov/ij). Quantitative analysis of parasite length was performed on male worms as male schistosomes always outnumber females in experimental infections and female growth is significantly influenced by pairing with males (Hernandez et al., 2004). Liver tissue was digested in 0.7% trypsin (50 ml) in PBS for 2–3 h at 37 °C and eggs were counted under a dissecting microscope.

Parasite antigen preparation

For preparation of soluble worm antigen (SWAP), adult *S. mansoni* worms (male and female) were suspended in ice-cold PBS and homogenized on ice. Insoluble material was removed by centrifugation at 13,200 x g for 45 min at 4°C, and the supernatant was filter-sterilized. Protein concentration was determined by the Bradford assay (BioRad, Hercules, CA) on a Spectramax M2 micro plate spectrophotometer (Molecular Devices, Sunnyvale, CA) and frozen in aliquots at –80°C.

$CD4^+$ T cell isolation and Cell culture

Single-cell suspensions of leukocytes were prepared from pooled spleens of infected mice by dissociating tissues with wash medium through nylon cell strainers and lysing erythrocytes with ACK lysing buffer if necessary. $CD4^+$ T cells were isolated by positive selection using magnetic anti-CD4 microbeads and MACS separation columns (Miltenyi

Biotech, CA) and following the instructions provided by the manufacturers. $CD4^+$ cells were cultured in T cell medium (TCM; RPMI 1640, 10% heat-inactivated FCS, 1000 U/ml penicillin, 10 μg/ml streptomycin, HEPES, 10mM; MEM Non-essential amino acids Solution, 10mM; L-glutamine, 200mM; Sodium pyruvate, 100mM; β-mercaptoethanol, 55mM) in the presence of $CD11c^+$ splenic dendritic cells (1:10 ratio of $CD4^+$ T cells: dendritic cells) isolated from non-infected C57BL/6 mice using magnetic anti-$CD11c^+$microbeads and MACS separation columns. SWAP was added to a final concentration of 50μg/ml. Positive and negative control cultures received 1μg/ml anti-CD3 antibody or no antigen, respectively. Following incubation for 72 h at 37°C in 5% carbon dioxide, supernatants were collected and immediately frozen at –80°C for subsequent determination of cytokine concentration.

Cytokine analysis

The concentrations of cytokines in culture supernatants and plasma were determined using sandwich ELISA kits from BD Biosciences/Pharmingen (San Diego, CA), following the instructions issued by the manufacturer. Briefly, for each cytokine, flat-bottomed plates (Immulon 2HB; Thermo, MA) were each coated overnight at 4 °C with the appropriate capture antibody diluted in carbonate-bicarbonate coating buffer (pH 9.5). Following an initial wash with PBS (pH 7.2) containing 0.05% Tween 20 (PBST; Sigma), the plates were blocked with 10% fetal calf serum (FCS) in PBS for 1 h at room temperature. Samples or standards were then added to the wells and the plates were incubated for 2 hours at RT. After incubation, the plates were washed and 100 μl of

working detector containing biotinylated anti-mouse cytokine antibody and Streptavidin–HRP (horseradish peroxidase conjugate) was added in a one-step incubation for 1 hour. After 7-10 washes, the substrate solution TMB and hydrogen peroxide was added to each well and 30 minutes after incubation in the dark, the reaction was stopped with 2N H_2SO_4 Absorbance was read at 450 nm using a Spectramax M2 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) and corrected for plate absorbance at 570 nm. Concentrations of cytokines (pg/ml) in the samples were determined by interpolation from standard curves calculated for each plate using appropriate amounts of recombinant mouse cytokines.

Antibodies

Mouse mAb to TGF-β (IgG1, clone ID11; American Type Culture Collection); and mouse ab to light harvesting chlorophyll a/b protein complex (IgG1, clone MLH2) were affinity purified from culture supernatant using protein G and fast performance liquid chromatography (Akta FPLC; Amersham Biosciences). The column was equilibrated with 0.02 M sodium phosphate (pH 7.0), and bound protein was eluted with 0.1 M glycine-HCL (pH 2.7). Fractions were neutralized with 1 M Tris-HCL (pH 9.0). Purified antibody was exchanged into PBS using a HiPrep 26/10 desalting column (Amersham Biosciences), filter sterilized and frozen in aliquots at –80°C.

In vivo Ab treatments

Mice (wild type and IL-$10^{-/-}$) were administered 1 mg of neutralizing Ab to TGF-β or control i.p (intra peritoneal) each week beginning a week prior to infection. Mice were infected on day 0 and sacrificed at 6 weeks post infection. Blood was collected from each mouse at 6 weeks post infection and serum frozen at –80°C.

ELISA for mouse immunoglobulin isotypes

Enzyme-linked immunosorbent assays was used to study antibody isotype responses of infected mice to crude schistosome antigens. 4HBX plates (Immulon Thermo, MA) were coated with SWAP (5μg /ml) in borate buffered saline (BBS) overnight at 4 ^{0}C. After 5 washes and blocking with BBS containing 1% of fetal calf serum, the immune sera were serial diluted in BBS containing 0.02% Tween 20 (BBST; Sigma, St Louis) and applied to the plates for 2 hours at RT. After 5 washes, the plates were incubated with alkaline phosphatase-conjugated goat antibodies against mouse IgG1, IgG2a, and IgG2b (Southern Biotechnology Associates), diluted 1:1000 in BBST for 30 minutes at RT and then washed 10 times. The reaction was developed by addition of 4- methylumbelliferyl phosphate substrate (4-MUP; Sigma). The fluorescence was detected on SPECTRAmax M2 fluorometer at excitation and emission wavelengths of 360nm and 449 nm, respectively.

Statistical analysis

Because unequal variances were observed among some of the groups analysed in this study, stringent non-parametric tests were used throughout to test the significance of differences between experimental groups. For two groups, significance of differences between experimental groups was tested using Mann–Whitney tests, and for three groups the significance of differences was tested using Kruskal–Wallis tests followed by Dunns' multiple comparison tests. Statistical analyses were performed with GraphPad Prism Version 4.0 software (GraphPad Software, Inc., San Diego, CA). *P* values of less than 0.05 were considered significant. All experiments were performed twice with similar results.

Results

IL-10 and TGF-β modulate IFN-γ production in *S. mansoni* infection

To test if absence of both IL-10 and TGF-β will have a stimulatory effect on cytokine production in a schistosomiasis model, IL-$10^{-/-}$ mice were infected with *S. mansoni* cerceriae and TGF-β was neutralized *in vivo* by weekly administration of TGF-β antibody. Six weeks later, $CD4^{+}$ T cells were isolated from spleens of both groups and stimulated *in vitro* with dendritic cells and SWAP. When compared to IL-$10^{-/-}$ mice that received isotype control antibody, IL-$10^{-/-}$ mice that received TGF-β antibody treatment produced significantly higher amounts of IFN-γ (Fig. 15A). However when the same treatment was repeated in wild type mice, absence of TGF-β alone did not cause a significant difference in IFN-γ production compared to wild type mice receiving isotype control antibody (Fig. 15B). Moreover, IL-4 production (Fig. 15C) and IL-10 levels (Fig. 15D) were also not affected by absence of TGF-β alone. Thus TGF-β plays a role in regulating IFN-γ responses to schistosome infection in the absence of IL-10, but not when IL-10 responses are intact. Interestingly, the weight of livers from the IL-$10^{-/-}$ treated with TGF-β antibody was increased compared to wild type mice treated with isotype control antibody (Fig. 15E), likely indicating an increase in cellular infiltration due to the disruption of both IL-10 and TGF-β regulatory mechanisms.

Modulation of immunoglobulin isotype switching in IL-$10^{-/-}$ mice treated with TGF-β antibody

Immunoglobulin isotype switching by B lymphocytes to IgG1 and IgG2a production is stimulated by T cell help from IL-4-producing Th2 cells and IFN-γ-producing Th1 cells, respectively. To investigate if the treatment of infected IL-$10^{-/-}$ mice with TGF-β antibody resulted in potentiation of schistosome-induced Th1 responses, we measured plasma concentrations of antibody isotypes specific for schistosome worm antigens. Our results show that SWAP-specific IgG2a (Fig. 16A) levels were significantly elevated in IL-$10^{-/-}$ mice treated with TGF-β antibody when compared to wild type mice that received isotype control antibody, suggesting that TGF-β plays a compensatory role in regulating worm-induced Th1 responses in the absence of IL-10. In contrast, similar levels of worm-specific IgG1 were produced by all groups (Fig. 16B), indicating that neither IL-10 nor TGF-β regulate schistosome worm-induced Th2 responses during early infection. SWAP-specific IgG2b levels were also measured, as there is limited *in vitro* evidence to suggest that class-switching to IgG2b is stimulated by TGF-β (Snapper, 1999). However, we could find no evidence of TGF-β mediated stimulation of class-switching to IgG2b, as anti-SWAP IgG2b levels were not affected by TGF-β neutralization in wild type mice and were elevated in anti-TGF-β-treated IL-10-/- mice (Fig. 16C). Thus, TGF-β plays a compensatory role in regulating worm-induced Th1 responses in the absence of IL-10, but not in the presence of intact IL-10 signaling.

Absence of IL-10 and TGF-β affects *S. mansoni* worm development but does not protect against infection

We next assessed whether simultaneous neutralization of TGF-β and IL-10 had a protective effect against primary schistosome infection, by preventing parasite establishment or interfering with development of the parasite. The numbers of worms recovered from the IL-$10^{-/-}$/anti-TGF-β group were not significantly different from the WT/isotype control group (Fig. 17A) indicating that there was no protection exhibited against schistosome infection in the absence of IL-10 and TGF-β.

To determine whether simultaneous neutralization of IL-10 and TGF-β affected worm growth and development, we measured the lengths of the parasites recovered from treated mice as well as the number of eggs deposited by worm pair. Although there was no difference in the mean value of worm length (Fig. 17B) between the IL-$10^{-/-}$/TGF-β and WT/isotype control groups, there was a significant difference ($P < 0.0001$) in the variance of the data obtained from each group (Fig. 17B), suggesting that neutralization of both IL-10 and TGF-β modulated schistosome growth in some way and resulted in the persistence of abnormally small worms in the IL-$10^{-/-}$/TGF-β group. However, egg production was similar between all groups (Fig. 17C). These results suggest that the absence of both IL-10 and TGF-β can influence on the development of *S. manson*i worms, but that neutralization of both cytokines has no protective effect against infection and that reproductive fitness of the parasites that reach maturity is not compromised.

Discussion

Our previous work demonstrated that schistosome infection induces a regulatory response by $CD4^+$ T cells to worm antigens that begins in early infection (Makarem and Davies, submitted). Since regulation of the immune system allows for control of over-exuberant responses to invading organisms to avoid host damage, we hypothesized that schistosomes co-opt this mechanism as a strategy to evade the host immune response. To test this hypothesis, we examined the establishment and development of *S. mansoni* parasites in the absence of regulatory mechanisms. Neutralization of TGF-β in IL-$10^{-/-}$ mice potentiated IFN-γ responses by $CD4^+$ T cells after re-stimulation with *S. mansoni* worm antigens, suggesting that dysregulation of worm-induced Th1 responses occurs when both regulatory cytokines are absent (Fig. 15). Neutralization of TGF-β in IL-$10^{-/-}$ mice resulted in an increase in liver weights, presumably due to an increase in cellular infiltration in the absence of both regulatory cytokines. Further evidence of Th1 dysregulation during schistosome infection was provided by the demonstration of augmented IgG2a responses to worm antigens in the absence of both IL-10 and TGF-β (Fig. 16). However, neutralization of TGF-β in wild type mice did not influence the production of IFN-γ, IL-4, or IL-10, or significantly alter organ weight or isotype responses, suggesting that TGF-beta plays a compensatory role in regulating $CD4^+$ T cell reponses in the absence of IL-10, but is of limited importance when IL-10 signaling is intact (Fig. 15).

To understand the effect of the lack of IL-10 and TGF-β on *S. mansoni* parasites, we evaluated the worm burden and parasite phenotype in IL-$10^{-/-}$ mice where TGF-β was neutralized. In previous studies where this approach was employed in a *Trichinella*

spiralis model of infection, neutralization of TGF-β in IL-$10^{-/-}$ mice resulted in a significant reduction in larval worm burdens (Beiting et al., 2007). In contrast, we found that simultaneous abrogation of IL-10 and TGF-β signaling did not inhibit the establishment of *S. mansoni* worms. Interestingly however, *S. mansoni* development was affected by simultaneous TGF-β and IL-10 inhibition, with significantly greater variance evident in the worm length data obtained from anti- TGF-β-treated IL-$10^{-/-}$ mice (Fig. 17), suggesting that interfering with the regulatory response has an effect on the development of this parasite. Taken together, these results suggest that the lack of immune regulation and resulting augmentation of immune responses, caused by loss of both TGF-β and IL-10 signaling (Beiting et al., 2007), contributed to the effect on worm development we observed. Hence there is likely redundancy between TGF-β and IL-10, such that an effect on schistosome development is only evident when both cytokines are absent. We hypothesize that simultaneous inhibition of other regulatory mechanisms, such as adenosine (Hasko and Cronstein, 2004) or CTLA-4 signaling (Hodi, 2007), may interfere further with the development of schistosome infections and could ultimately result in a decrease in worm burdens.

An alternative explanation for our results may be that schistosomes utilize host TGF-β as a developmental signal during the early stages of infection. This possibility is supported by the observations that (i) schistosome worms express members of the TGF-β receptor family (Davies et al., 1998b), (ii) that these receptors may be exposed on the parasite surface and therefore available for binding host cytokines (Davies et al., 1998b), and (iii) there is molecular evidence that the schistosome receptors can interact functionally with mammalian TGF-β (Beall and Pearce, 2001). However, neutralization

of TGF-β alone, in IL-10-expressing wild type mice, did not affect parasite development, arguing that the alterations in parasite development were instead due to increased immunological damage to the parasites when two important regulatory cytokines are neutralized.

Interestingly, no impairment of class switching to IgG2b production was observed in either wild type or IL-$10^{-/-}$ mice when treated with anti-TGF-β antibody, despite the fact that TGF-β was proposed to play a role in signaling B cells to switch to IgG2b production, at least *in vitro* (Snapper, 1999). Our data suggest that TGF-β has little or no role to play in immunoglobulin isotype switching *in vivo*. Indeed, in many *in vivo* studies, IgG2b production appears to closely parallel IgG2a production.

In conclusion, our data suggest that schistosomes induce regulatory responses during the early stages of infection to avoid immune destruction and facilitate establishment within the host. Consequently, inhibition of schistosome-induced regulatory mechanisms may interfere with the ability of schistosomes to evade immune destruction. Strategies aimed at disrupting schistosome-induced regulatory mechanisms could therefore provide a useful adjunct to anti-schistosome therapies such as praziquantel, which require immune responses to eliminate schistosomes (Brindley and Sher, 1987; Brindley et al., 1989) and are rarely if ever 100% efficacious (King, 2006) . Further, inhibition of schistosome-induced regulatory responses could potentiate the protective capabilities of existing and future anti-schistosome vaccines (Pearce, 2003), potentially leading to the development of vaccines with sufficient efficacy to be useful in controlling schistosome infection in human populations.

Figure 13: Treatment of IL-$10^{-/-}$ mice with TGF-β antibody increases IFN-γ production by $CD4^+$ T cells. (A) IL-$10^{-/-}$ mice were treated with either anti-TGF-β antibody (n = 4) or isotype control antibody (n = 3), and infected with *S. mansoni* cerceriae. Six weeks later, $CD4^+$ T cells were isolated from spleens, cultured with dendritic cells ($2x10^6$ $CD4^+$ T cells:200,000 DC's) and stimulated with SWAP. IFN-γ cytokine level was measured from supernatants 72 h after stimulation ($p< 0.05$). (B-D) Splenic $CD4^+$ T cells from wild type mice (n = 5 both groups) that received the same antibody treatments and infected for the same amount of time were isolated, cultured with dendritic cells and stimulated with SWAP. IFN-γ (B), IL-4 (C), and IL-10 (D) cytokine levels were measured from supernatants 72 h after stimulation ($p> 0.05$). Liver weights (E) from individual IL-$10^{-/-}$ and wild type mice treated with either anti-TGF-β antibody or isotype control antibody, was measured 6 weeks after infection.

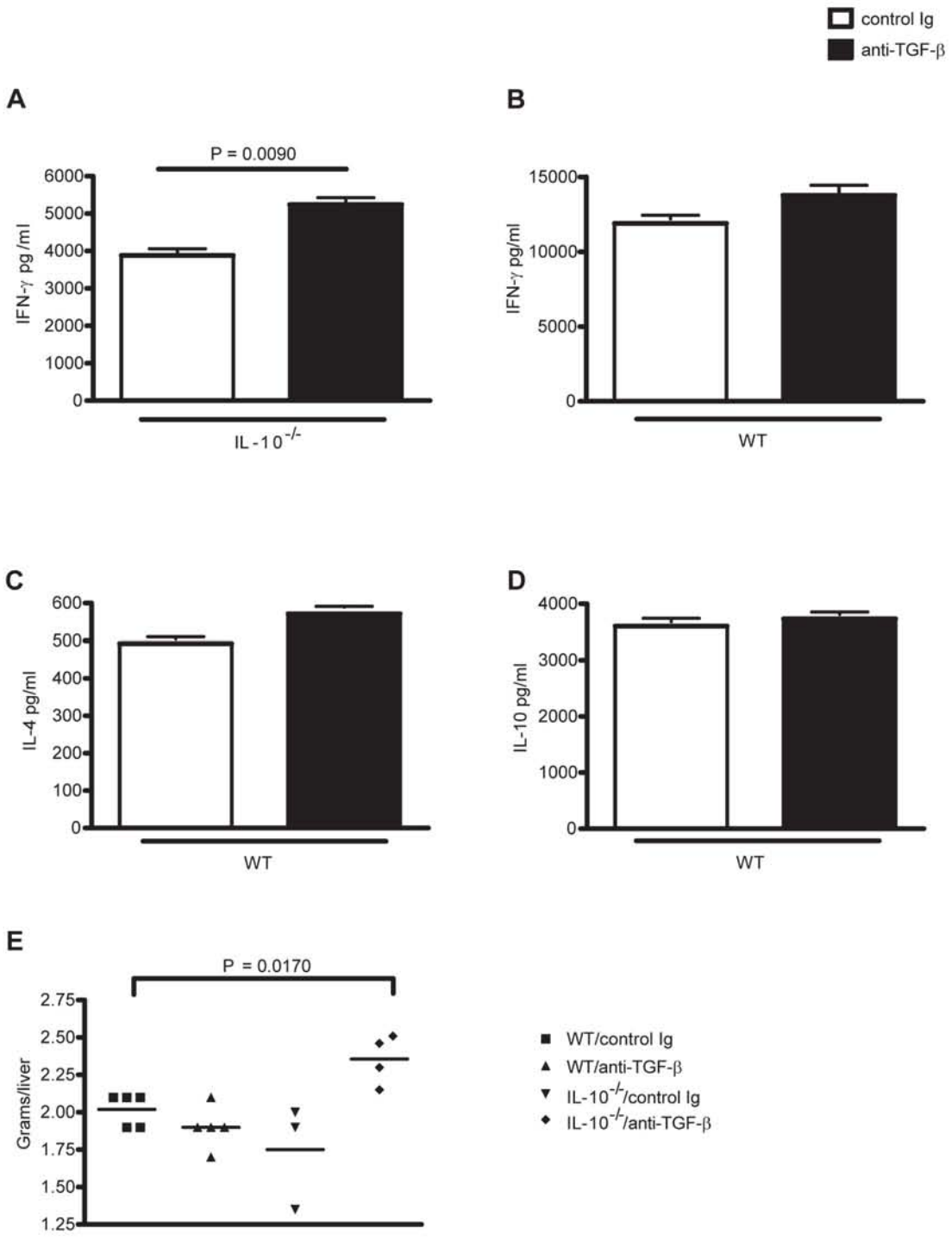

control Ig
anti-TGF-β
A
P = 0.0090
IFN-γ pg/ml
6000
5000
4000
3000
2000
1000
0
IL-10-/-
B
IFN-γ pg/ml
15000
10000
5000
0
WT
C
IL-4 pg/ml
600
500
400
300
200
100
0
WT
D
IL-10 pg/ml
4000
3000
2000
1000
0
WT
E
P = 0.0170
Grams/liver
2.75
2.50
2.25
2.00
1.75
1.50
1.25
WT/control Ig
WT/anti-TGF-β
IL-10-/-/control Ig
IL-10-/-/anti-TGF-β

Figure 14: Enhancement of *S. mansoni*-induced Th1 responses in IL-$10^{-/-}$ mice by TGF-β neutralization. Both IL-$10^{-/-}$ and wild type mice were treated with either anti-TGF-β antibody or isotype control antibody and infected with *S. mansoni* cerceriae. Six weeks later, blood was collected from each mouse and serum was used to measure SWAP-specific IgG2a (A), IgG2b (B) and IgG1 (C) antibody isotype levels. Sera from non-infected wild type animals were included as negative controls.

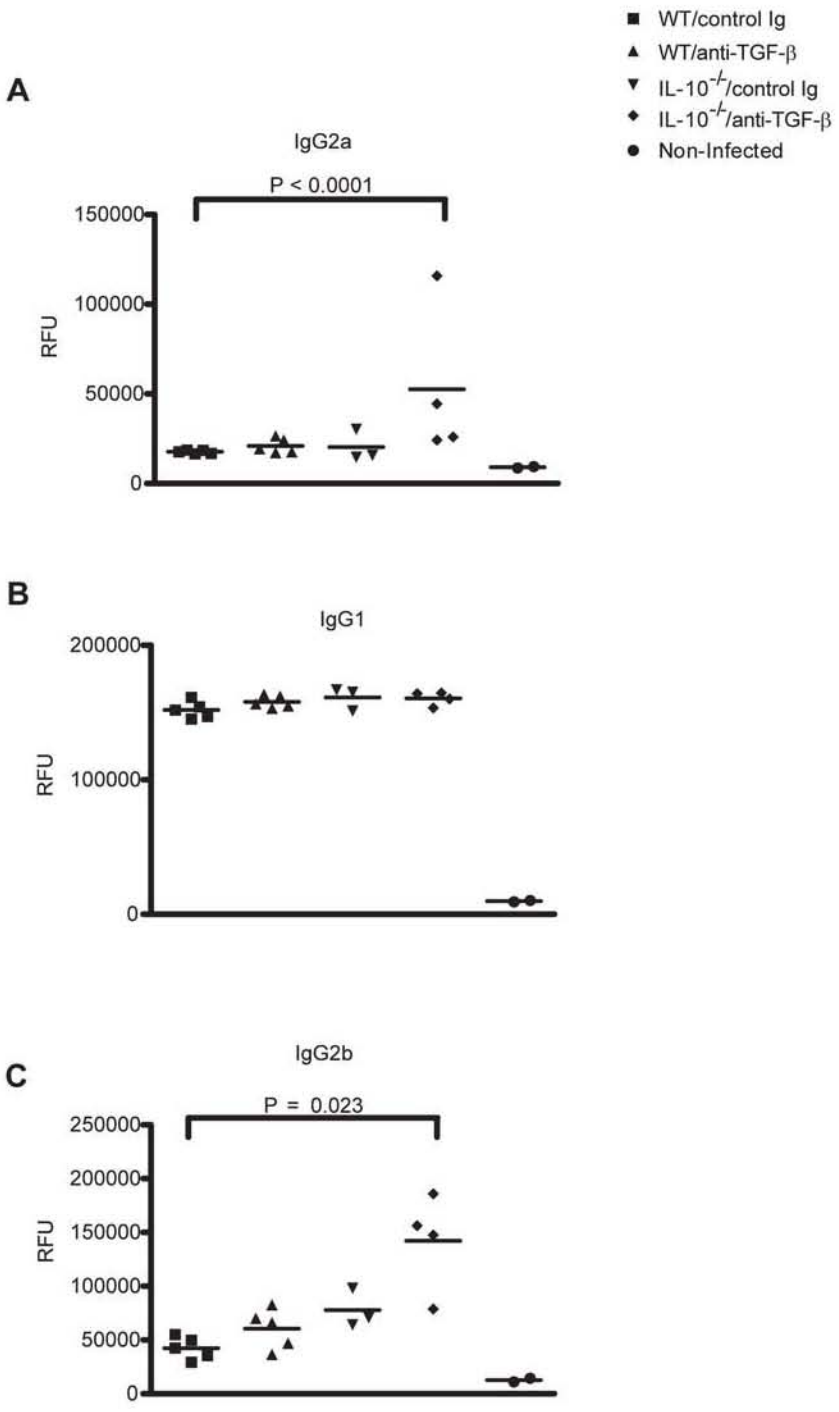
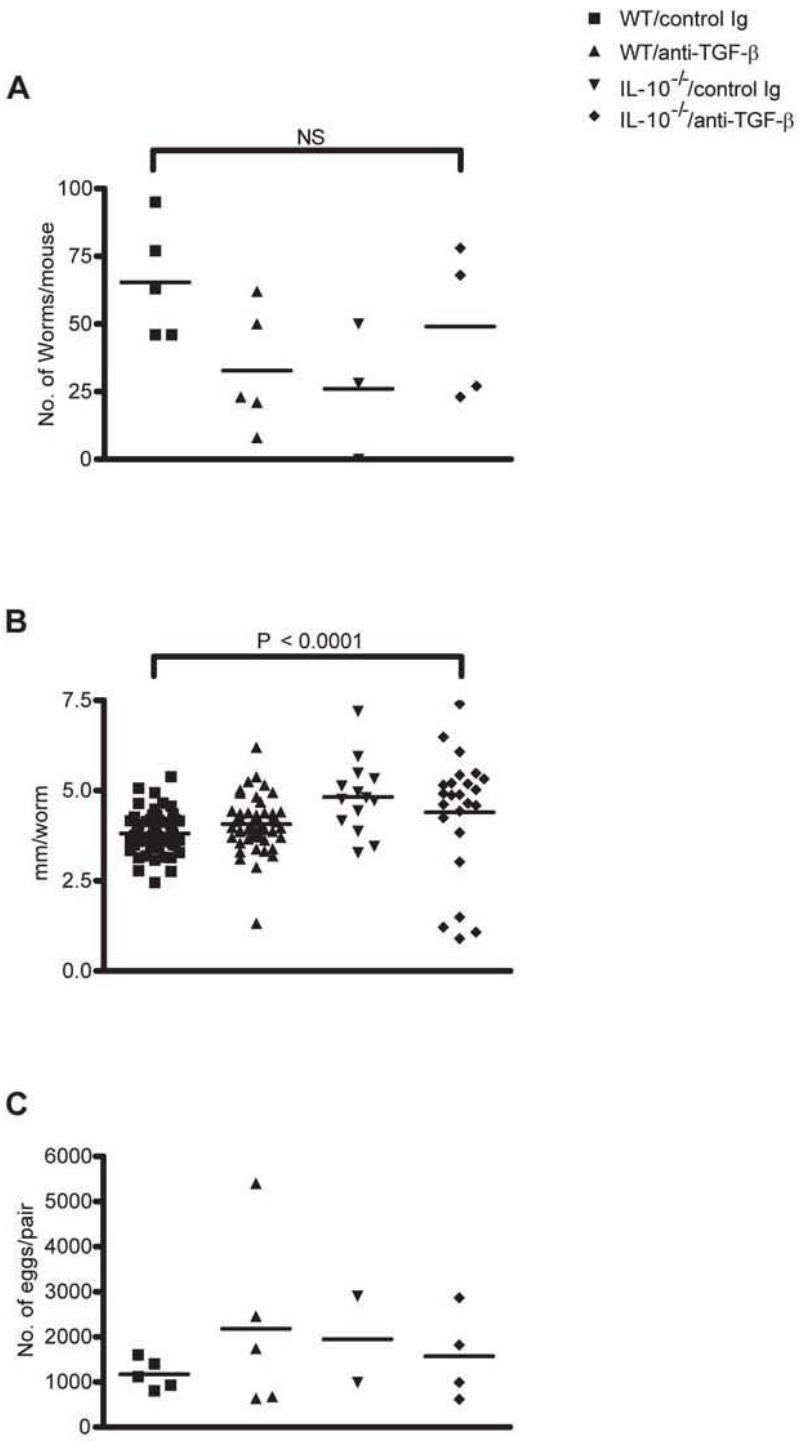

WT/control Ig
WT/anti-TGF-β
IL-10-/-/control Ig
IL-10-/-/anti-TGF-β
Non-Infected
A
IgG2a
P < 0.0001
RFU
150000
100000
50000
0
B
IgG1
RFU
200000
100000
0
C
IgG2b
P = 0.023
RFU
250000
200000
150000
100000
50000
0

Figure 15: Development of *S. mansoni* worms is affected by absence of IL-10 and TGF-β. Number of worms (A), worm length (B), and eggs per worm pair (C) from individual IL-$10^{-/-}$ and wild type mice treated with either anti-TGF-β antibody or isotype control antibody, was assessed 6 weeks after infection. In (B), the *P* value was calculated using the *F* test to compare the variances between the two groups.


WT/control Ig
WT/anti-TGF-β
IL-10-/-/control Ig
IL-10-/-/anti-TGF-β
A
NS
No. of Worms/mouse
100
75
50
25
0
B
P < 0.0001
mm/worm
7.5
5.0
2.5
0.0
C
No. of eggs/pair
6000
5000
4000
3000
2000
1000
0

Chapter 4

Summary and Future Directions

Systemic regulatory $CD4^+$ T cell response to schistosome worm antigens

In Chapter 2, we show that in a murine model of schistosomiasis, primary infection with *S. mansoni* worms leads to production of the regulatory cytokine IL-10 by schistosome antigen-specific $CD4^+$ T cells, which peaks at 4 weeks post-infection before egg deposition begins. High levels of IL-10 mRNA synthesis by $CD4^+$ T cells were detected in the liver, while IL-10 protein production in response to SWAP was seen in $CD4^+$ T cells from both liver and spleen. Interestingly, IFN-γ protein levels from hepatic $CD4^+$ T cells stimulated with SWAP were negligible in wild type mice but increased dramatically in IL-$10^{-/-}$ mice, indicating that IL-10 contributes to the suppression of the early IFN-γ response. This finding could perhaps explain why the T helper response prior to the onset of egg deposition has been characterized as a limited Th1 response (Pearce and MacDonald, 2002). Our data suggest that, in the context of an intact immune system, this response is best characterized as a regulatory T cell response.

We hypothesize that production of IL-10 is induced by the parasite early in infection to evade the pro-inflammatory cytokines that might be detrimental to its survival and establishment in the host. This hypothesis is supported by evidence from previous work in humans that showed elevated levels of IFN-γ production by $CD4^+$ T cells to worm antigens in resistant but exposed patients living in endemic areas (Endemic Normals; EN) (Brito et al., 2000). IL-10 secretion in response to worm antigens in EN individuals was found to be decreased, but not significantly so, when compared to non-infected, treated, or chronically infected patients. However, neutralizing IL-10 *in vitro* produced high levels of IFN-γ to SWAP only in cells from EN individuals compared to the other groups, indicating that individuals resistant to infection have the propensity to

mount a Th1 type immune response as a result of lower IL-10 levels (Brito et al., 2000). Thus, there appears to be a correlation between resistance to schistosome infection in human patients and a change in the balance of IL-10 and IFN-γ production that favors production of IFN-γ. However, further studies involving more individuals are needed to test this potential correlation further.

Although our data provide evidence that schistosomes induce IL-10 in early infection, leading to lower levels of IFN-γ and impairing pro-inflammatory responses against the invading parasites, normal schistosome establishment and development is seen during primary infection of IL-$10^{-/-}$ mice (Wynn et al., 1998). Therefore, neutralizing IL-10 alone is not sufficient for protection against a primary schistosome infection. However, published data suggest that IL-10 interferes with vaccine-induced immunity to schistosomes and that vaccinating IL-$10^{-/-}$ mice results in greater protection to schistosome infection (Hoffmann et al., 1999). A vaccine that induces a strong Th1 response, while avoiding induction of IL-10 production, could therefore be more effective in inducing protection against re-infection. Before this can be achieved, the mechanisms by which schistosome worms induce the production of IL-10 must be determined, so that vaccines that do not activate these mechanisms can be developed. It is interesting to note that the best current experimental vaccine, utilizing irradiated cercariae, still induces IL-10 production and this could explain why this vaccine is not more efficacious. We speculate that specific schistosome antigens may be responsible for inducing IL-10 production. Indeed, preliminary data suggest that immunodominant antigens recognized by the early response to schistosome worms may also be the primary targets of IL-10 responses (Fraga, Makarem and Davies, in preparation). These

immunodominant antigens include the secreted schistosome gut proteases Sm31 and Sm32 (Caffrey et al., 2004). Whether the proteolytic activity of these antigens is linked to their immunogenicity or their capacity to induce IL-10 is currently under investigation.

Interestingly, IL-10 has also been attributed to the down-modulation of hepatic granuloma formation at the acute stage of infection (Hesse et al., 2004; Hoffmann et al., 2000), leading to a decrease in hepatic granuloma size and fibrosis, which is in turn beneficial to host survival. However, it is not particularly clear if IL-10 is involved in controlling granulomatous inflammation at the chronic phase of the infection since it has been shown that IL-$10^{-/-}$ mice undergo normal down-modulation of granuloma formation (Wynn et al., 1998). Since there is a possibility that other factors may be involved in the down-modulation of hepatic granuloma formation and fibrosis, it would be interesting to assess whether adenosine signaling through the A2A receptor plays a part in this down-modulation at the chronic stage of infection. Our data show that fibrosis is increased in $A2AR^{-/-}$ mice at the acute phase of the infection (8 weeks post-infection, Fig. 8), but there was no difference in granuloma size (data not shown). Therefore, assessing granuloma size and fibrosis in $A2AR^{-/-}$ mice 16-20 weeks post-infection (chronic infection) will lead us to a better understanding of whether adenosine signaling is involved in down-modulating hepatic granuloma formation and fibrosis at the chronic phase of the infection.

Since $A2AR^{-/-}$ mice exhibited more fibrosis than wild type mice, this suggests that stimulation of A2AR signaling rather than its inhibition may be beneficial in alleviating the fibrosis in schistosome infection. An interesting experiment would be to administer the A2AR agonist to wild type mice and assess the fibrosis 8 weeks post-infection.

Comparing this group to another group receiving an A2AR inhibitor, we expect to see more fibrosis in the mice that receive the A2AR agonist since the abrogation of A2AR in the $A2AR^{-/-}$ mice lead to an increase in hepatic fibrosis. This will further clarify the role of adenosine signaling in schistosome infections, and could be exploited as a possible therapy for reducing fibrosis in schistosomiasis.

Effects of $CD4^+$ T cell antigen specificity on schistosome worm development

Previous studies have shown that $CD4^+$ T cells are critical to the normal development of schistosome worms during the early phase of infection prior to egg laying (Davies et al., 2001; Lamb et al., 2007). Indeed, in immunodeficient mice such as SCID or $RAG^{-/-}$ mice, schistosomes grow much more slowly during the prepatent period than they do in immunocompetent mice, the worms take longer to form pairs, and their reproductive fitness is impaired, as demonstrated by their greatly reduced rates of egg production (Davies et al., 2001). Remarkably, reconstitution of $RAG^{-/-}$ mice with $CD4^+$ T cells alone is sufficient to restore parasite development and reproductive capacity to normal levels (Davies et al., 2001; Lamb et al., 2007). However, little is currently known regarding how $CD4^+$ T cells mediate these effects on schistosome development. Because the unique and primary function of $CD4^+$ T cells is to respond to foreign peptides of extracellular origin that are presented to them by MHC class II-expressing cells, we hypothesized that the responses of $CD4^+$ T cells to schistosomes during the prepatent period were important for the positive effects that $CD4^+$ T cells have on developing schistosomes. To examine this hypothesis, we tested whether adoptive transfer of $CD4^+$ T cells that were previously primed by schistosome worm antigens would be more effective than naïve cells at restoring worm development in $RAG^{-/-}$ recipients. However, we found no differences between naïve and primed $CD4^+$ T cells in their ability to facilitate worm development, regardless of whether the cells were primed by previous infection or vaccination, suggesting that the specificity and effector function of $CD4^+$ T cells has little or no impact on schistosome development. These findings are in agreement with other

data showing that $CD4^+$ T cells with defects in T cell receptor-mediated activation or with specificity for completely irrelevant antigens can also facilitate schistosome development (Lamb and Davies, submitted). Together, these data suggest that the mechanisms by which $CD4^+$ T cells facilitate blood fluke development are separate and distinct from their antigen responsiveness and effector function. It is encouraging that distinctions can be drawn between the positive effects of $CD4^+$ T cells on schistosome development on one hand and the effector functions of these cells on the other, as it suggests that priming of $CD4^+$ T cells for anti-schistosome effector functions by vaccines may be possible without inadvertently enhancing schistosome worm development.

In agreement with the findings outlined above, we also found that in a murine vaccination model that elicits partial protection (Hewitson et al., 2005), the worms that escape immune destruction develop normally. This indicates that even when $CD4^+$ T cells mediate protection, they also simultaneously facilitate schistosome worm development. A strategy that may improve the efficacy of future vaccines might be to attempt inhibition or disruption of interactions between host $CD4^+$ T cells and schistosomes that facilitate parasite development, while leaving protective effector functions of $CD4^+$ T cells intact. Because $CD4^+$ T cells play a central role in mediating immunity to a plethora of pathogens, broad inhibition of T cell function to disrupt schistosome development is not desirable. Before selective inhibition or disruption of specific schistosome-T cell interactions can be rationally implemented, identification and molecular characterization of the interactions that have a positive effect on schistosome development is essential.

One caveat of our results in comparing the transfer of primed or naïve $CD4^+$ T cells into $RAG^{-/-}$ mice is that due to homeostatic proliferation in the $RAG^{-/-}$, the naïve $CD4^+$ T cells acquire the same phenotype as that of primed $CD4^+$ T cells. Therefore, comparing primed or naïve $CD4^+$ T cells on their ability to facilitate worm development, is difficult to assess. To overcome this problem, future experiments should explore the possibility of transferring primed and naïve $CD4^+$ T cells into mice that lack the T cell receptor α/β only. These $TCR^{-/-}$ mice are not able to mount $CD4^+$ T cell responses, but have intact B and $\gamma\delta$ T cell compartments and therefore do not support such extensive homeostatic proliferation. Avoiding the impact of homeostatic proliferation on our transfer experiments will help us to better assess the effect of primed or naïve $CD4^+$ T cells on their ability to facilitate worm development.

TGF-β and IL-10 in schistosome worm growth and development

Because our data clearly show that a regulatory T cell response to schistosome worms is induced during the early stages of schistosome infection, we hypothesized that this represents an important immune evasion strategy for the parasite, allowing it to prevent potentially damaging effector responses and escape immune destruction. However, IL-10-deficient mice are as susceptible to primary schistosome infection as wild type mice (Wynn et al., 1998) and worm development proceeds normally in these animals, leading us to hypothesize that other regulatory mechanisms can compensate for the loss of IL-10 in IL-$10^{-/-}$ mice. One potential redundant mechanism that could play this compensatory role is the cytokine TGF-β, which was previously shown to inhibit immune elimination of worms in a murine model of *Trichinella spiralis* infection (Beiting et al., 2007). We therefore evaluated the role of redundancy in regulatory responses as a potential strategy used by schistosomes to evade protective immune responses in early infection by inhibiting IL-10 and TGF-β signaling simultaneously. Our data show that in an *in vivo* context, neutralizing TGF-β in IL-$10^{-/-}$ mice causes an increase in pro-inflammatory responses and has an inhibitory effect on *S. mansoni* growth. This effect was not observed when either TGF-β or IL-10 was absent alone, indicating that there is redundancy in TGF-β and IL-10 regulatory functions. Furthermore, there are potentially other regulatory mechanisms (Belkaid, 2007) that may be induced by schistosomes during early infection, resulting in simultaneous activity of multiple, functionally overlapping regulatory mechanisms that keep protective effector mechanisms in check. Inhibition of several regulatory mechanisms at once, for example by neutralizing IL-10 and TGF-β, blocking adenosine signaling by A2AR inhibitors, and

blocking CTLA-4 signaling, may therefore represent a strategy for enhancing the efficacy of treatments and vaccinations aimed at eliminating or preventing schistosome infection. However, simultaneous inhibition of multiple immune regulatory mechanisms runs the risk of allowing excessive inflammation or autoimmune responses to occur (Belkaid, 2007), with potentially serious implications for the health of the host, and should be taken into consideration.

Multiple components of a functional TGF-β pathway, including different TGF-β receptor family members, have been characterized on the surface of *S. mansoni* worms (Forrester et al., 2004; Osman et al., 2001; Osman et al., 2006). These include receptors that may be important in worm development and a recently identified TGF-β family ligand that may play a crucial role in egg development (Freitas et al., 2007). Because of the surface localization of some the TGF-β family receptors, it has been hypothesized that the ligands for schistosome TGF-β receptors are of host origins (Beall and Pearce, 2001, 2002). Importantly, there is also a possibility that TGF-β ligands from schistosome worms could act as ligands to initiate signaling in host cells, leading to production of further host TGF-β and establishment of a positive feedback mechanism (Freitas et al., 2007). Therefore, in addition to neutralizing host TGF-β, blocking schistosome TGF-β pathway components by using RNAi strategies to knockdown TGF-β receptor genes on schistosomes such as SmTβ RI (Davies et al., 1998a) and SmTβ RII (Osman et al., 2006), could result in multiple detrimental effects on the parasite and may be of practical use in controlling schistosome infections.

Concluding Remarks

In summary, we show that while schistosome infection rapidly induces a regulatory immune response, the phenotype and specificity of the $CD4^+$ T cell response does not appear to play a crucial role in providing the positive signals that schistosomes need from $CD4^+$ T cells to complete their development normally. These findings point the way to a novel strategy for developing new and more effective therapeutic and prophylactic measures for combating schistosomiasis. This strategy will combine inhibition of regulatory mechanisms to counter immune evasion by schistosomes, while also augmenting effector mechanisms that eliminate schistosomes, which should be possible without potentiating positive signals for parasite development. Implementation of this approach will first require molecular characterization of the mechanisms by which schistosomes induce regulatory responses and by which the immune system most effectively eliminates schistosomes. While there is considerable data in the literature on the latter mechanisms (Hewitson et al., 2005), the former remain obscure. Finally, molecular characterization of the mechanisms by which $CD4^+$ T cells facilitate parasite development may identify additional host-parasite interactions that can be manipulated to benefit the host, providing a three-pronged approach to managing a pathogen that evades and co-opts immune function with remarkable success.

Refrences

Abath, F.G., Morais, C.N., Montenegro, C.E., Wynn, T.A., and Montenegro, S.M. (2006). Immunopathogenic mechanisms in schistosomiasis: what can be learnt from human studies? Trends Parasitol *22*, 85-91.

Amiri, P., Locksley, R.M., Parslow, T.G., Sadick, M., Rector, E., Ritter, D., and McKerrow, J.H. (1992). Tumour necrosis factor alpha restores granulomas and induces parasite egg-laying in schistosome-infected SCID mice [see comments]. Nature *356*, 604-607.

Anderson, C.F., Oukka, M., Kuchroo, V.J., and Sacks, D. (2007). CD4(+)CD25(-)Foxp3(-) Th1 cells are the source of IL-10-mediated immune suppression in chronic cutaneous leishmaniasis. J Exp Med *204*, 285-297.

Angeli, V., Faveeuw, C., Roye, O., Fontaine, J., Teissier, E., Capron, A., Wolowczuk, I., Capron, M., and Trottein, F. (2001). Role of the parasite-derived prostaglandin D2 in the inhibition of epidermal Langerhans cell migration during schistosomiasis infection. J Exp Med *193*, 1135-1147.

Awasthi, A., Carrier, Y., Peron, J.P., Bettelli, E., Kamanaka, M., Flavell, R.A., Kuchroo, V.K., Oukka, M., and Weiner, H.L. (2007). A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. Nat Immunol *8*, 1380-1389.

Basch, P.F. (1991). Schistosomes: Development, Reproduction and Host Relations (New York, Oxford University Press).

Baumgart, M., Tompkins, F., Leng, J., and Hesse, M. (2006). Naturally occurring CD4+Foxp3+ regulatory T cells are an essential, IL-10-independent part of the immunoregulatory network in Schistosoma mansoni egg-induced inflammation. J Immunol *176*, 5374-5387.

Beall, M.J., McGonigle, S., and Pearce, E.J. (2000). Functional conservation of Schistosoma mansoni Smads in TGF-beta signaling. Mol Biochem Parasitol *111*, 131-142.

Beall, M.J., and Pearce, E.J. (2001). Human transforming growth factor-beta activates a receptor serine/threonine kinase from the intravascular parasite Schistosoma mansoni. J Biol Chem *276*, 31613-31619.

Beall, M.J., and Pearce, E.J. (2002). Transforming growth factor-beta and insulin-like signalling pathways in parasitic helminths. Int J Parasitol *32*, 399-404.

Beiting, D.P., Gagliardo, L.F., Hesse, M., Bliss, S.K., Meskill, D., and Appleton, J.A. (2007). Coordinated control of immunity to muscle stage Trichinella spiralis by IL-10, regulatory T cells, and TGF-beta. J Immunol *178*, 1039-1047.

Belkaid, Y. (2007). Regulatory T cells and infection: a dangerous necessity. Nat Rev Immunol *7*, 875-888.

Belkaid, Y., Piccirillo, C.A., Mendez, S., Shevach, E.M., and Sacks, D.L. (2002). CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity. Nature *420*, 502-507.

Brindley, P.J., and Sher, A. (1987). Anti-schistosomal drugs: observations on the mechanism of drug resistance to hycanthone, and on the involvement of host antibodies in the mode of action of praziquantel. Mem Inst Oswaldo Cruz *82 Suppl 4*, 157-161.

Brindley, P.J., Strand, M., Norden, A.P., and Sher, A. (1989). Role of host antibody in the chemotherapeutic action of praziquantel against Schistosoma mansoni: identification of target antigens. Mol Biochem Parasitol *34*, 99-108.

Brito, C.F., Caldas, I.R., Coura Filho, P., Correa-Oliveira, R., and Oliveira, S.C. (2000). CD4+ T cells of schistosomiasis naturally resistant individuals living in an endemic area produce interferon-gamma and tumour necrosis factor-alpha in response to the recombinant 14KDA Schistosoma mansoni fatty acid-binding protein. Scand J Immunol *51*, 595-601.

Caffrey, C.R., McKerrow, J.H., Salter, J.P., and Sajid, M. (2004). Blood 'n' guts: an update on schistosome digestive peptidases. Trends Parasitol *20*, 241-248.

Chitsulo, L., Engels, D., Montresor, A., and Savioli, L. (2000). The global status of schistosomiasis and its control. Acta Trop *77*, 41-51.

Cioli, D. (1998). Chemotherapy of schistosomiasis: an update. Parasitol Today *14*, 418-422.

Colley, D.G., LoVerde, P.T., and Savioli, L. (2001). Infectious disease. Medical helminthology in the 21st century. Science *293*, 1437-1438.

Davies, S.J., Grogan, J.L., Blank, R.B., Lim, K.C., Locksley, R.M., and McKerrow, J.H. (2001). Modulation of Blood Fluke Development in the Liver by Hepatic CD4+ Lymphocytes. Science *294*, 1358-1361.

Davies, S.J., Shoemaker, C.B., and Pearce, E.J. (1998a). A divergent member of the transforming growth factor beta receptor family from Schistosoma mansoni is expressed on the parasite surface membrane. J Biol Chem *273*, 11234-11240.

Davies, S.J., Shoemaker, C.B., and Pearce, E.J. (1998b). A divergent member of the transforming growth factor beta receptor family from Schistosoma mansoni is expressed on the parasite surface membrane. J Biol Chem *273*, 11234-11240.

de Jesus, A.R., Magalhaes, A., Miranda, D.G., Miranda, R.G., Araujo, M.I., de Jesus, A.A., Silva, A., Santana, L.B., Pearce, E., and Carvalho, E.M. (2004). Association of type 2 cytokines with hepatic fibrosis in human Schistosoma mansoni infection. Infect Immun *72*, 3391-3397.

Deaglio, S., Dwyer, K.M., Gao, W., Friedman, D., Usheva, A., Erat, A., Chen, J.F., Enjyoji, K., Linden, J., Oukka, M., *et al.* (2007). Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. J Exp Med *204*, 1257-1265.

Fehervari, Z., and Sakaguchi, S. (2004). CD4+ Tregs and immune control. J Clin Invest *114*, 1209-1217.

Fontenot, J.D., and Rudensky, A.Y. (2005). A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. Nat Immunol *6*, 331-337.

Forrester, S.G., Warfel, P.W., and Pearce, E.J. (2004). Tegumental expression of a novel type II receptor serine/threonine kinase (SmRK2) in Schistosoma mansoni. Mol Biochem Parasitol *136*, 149-156.

Freitas, T.C., Jung, E., and Pearce, E.J. (2007). TGF-beta signaling controls embryo development in the parasitic flatworm Schistosoma mansoni. PLoS Pathog *3*, e52.

Georgi, J.R., Wade, S.E., and Dean, D.A. (1986). Attrition and temporal distribution of Schistosoma mansoni and S. haematobium schistosomula in laboratory mice. Parasitology *93 (Pt 1)*, 55-70.

Goldrath, A.W., Bogatzki, L.Y., and Bevan, M.J. (2000). Naive T cells transiently acquire a memory-like phenotype during homeostasis-driven proliferation. J Exp Med *192*, 557-564.

Harrop, R., and Wilson, R.A. (1993). Irradiation of Schistosoma mansoni cercariae impairs neuromuscular function in developing schistosomula. J Parasitol *79*, 286-289.
Hasko, G., and Cronstein, B.N. (2004). Adenosine: an endogenous regulator of innate immunity. Trends Immunol *25*, 33-39.

Herbert, D.R., Holscher, C., Mohrs, M., Arendse, B., Schwegmann, A., Radwanska, M., Leeto, M., Kirsch, R., Hall, P., Mossmann, H., *et al.* (2004). Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology. Immunity *20*, 623-635.

Hesse, M., Piccirillo, C.A., Belkaid, Y., Prufer, J., Mentink-Kane, M., Leusink, M., Cheever, A.W., Shevach, E.M., and Wynn, T.A. (2004). The pathogenesis of schistosomiasis is controlled by cooperating IL-10-producing innate effector and regulatory T cells. J Immunol *172*, 3157-3166.

Hewitson, J.P., Hamblin, P.A., and Mountford, A.P. (2005). Immunity induced by the radiation-attenuated schistosome vaccine. Parasite Immunol *27*, 271-280.
Hodi, F.S. (2007). Cytotoxic T-lymphocyte-associated antigen-4. Clin Cancer Res *13*, 5238-5242.

Hoffmann, K.F., Cheever, A.W., and Wynn, T.A. (2000). IL-10 and the dangers of immune polarization: excessive type 1 and type 2 cytokine responses induce distinct forms of lethal immunopathology in murine schistosomiasis. J Immunol *164*, 6406-6416.

Hoffmann, K.F., James, S.L., Cheever, A.W., and Wynn, T.A. (1999). Studies with double cytokine-deficient mice reveal that highly polarized Th1- and Th2-type cytokine and antibody responses contribute equally to vaccine-induced immunity to Schistosoma mansoni. J Immunol *163*, 927-938.

Hogg, K.G., Kumkate, S., Anderson, S., and Mountford, A.P. (2003a). Interleukin-12 p40 secretion by cutaneous CD11c+ and F4/80+ cells is a major feature of the innate immune response in mice that develop Th1-mediated protective immunity to Schistosoma mansoni. Infect Immun *71*, 3563-3571.

Hogg, K.G., Kumkate, S., and Mountford, A.P. (2003b). IL-10 regulates early IL-12-mediated immune responses induced by the radiation-attenuated schistosome vaccine. Int Immunol *15*, 1451-1459.

Homewood, C.A., Jewsbury, J.M., and Chance, M.L. (1972). The pigment formed during haemoglobin digestion by malarial and schistosomal parasites. Comp Biochem Physiol B *43*, 517-523.

Ivanov, II, McKenzie, B.S., Zhou, L., Tadokoro, C.E., Lepelley, A., Lafaille, J.J., Cua, D.J., and Littman, D.R. (2006). The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell *126*, 1121-1133.

James, S.L., Cheever, A.W., Caspar, P., and Wynn, T.A. (1998). Inducible nitric oxide synthase-deficient mice develop enhanced type 1 cytokine-associated cellular and humoral immune responses after vaccination with attenuated Schistosoma mansoni cercariae but display partially reduced resistance. Infect Immun *66*, 3510-3518.

James, S.L., and Glaven, J. (1989). Macrophage cytotoxicity against schistosomula of Schistosoma mansoni involves arginine-dependent production of reactive nitrogen intermediates. J Immunol *143*, 4208-4212.

James, S.L., Labine, M., and Sher, A. (1981). Mechanisms of protective immunity against Schistosoma mansoni infection in mice vaccinated with irradiated cercariae. I. Analysis of antibody and T-lymphocyte responses in mouse strains developing differing levels of immunity. Cell Immunol *65*, 75-83.

James, S.L., and Nacy, C. (1993). Effector functions of activated macrophages against parasites. Curr Opin Immunol *5*, 518-523.

James, S.L., Natovitz, P.C., Farrar, W.L., and Leonard, E.J. (1984). Macrophages as effector cells of protective immunity in murine schistosomiasis: macrophage activation in mice vaccinated with radiation-attenuated cercariae. Infect Immun *44*, 569-575.

James, S.L., Pearce, E.J., Lanar, D., and Sher, A. (1987). Induction of cell-mediated immunity as a strategy for vaccination against Schistosoma mansoni. Acta Trop Suppl *12*, 50-54.

Janeway, C.A. (2005). ImmunoBiology, 6th edn (Garland Science).

Jankovic, D., Cheever, A.W., Kullberg, M.C., Wynn, T.A., Yap, G., Caspar, P., Lewis, F.A., Clynes, R., Ravetch, J.V., and Sher, A. (1998). CD4+ T cell-mediated granulomatous pathology in schistosomiasis is downregulated by a B cell-dependent mechanism requiring Fc receptor signaling. J Exp Med *187*, 619-629.

Jankovic, D., Kullberg, M.C., Feng, C.G., Goldszmid, R.S., Collazo, C.M., Wilson, M., Wynn, T.A., Kamanaka, M., Flavell, R.A., and Sher, A. (2007). Conventional T-bet(+)Foxp3(-) Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection. J Exp Med *204*, 273-283.

Jankovic, D., Wynn, T.A., Kullberg, M.C., Hieny, S., Caspar, P., James, S., Cheever, A.W., and Sher, A. (1999). Optimal vaccination against Schistosoma mansoni requires the induction of both B cell- and IFN-gamma-dependent effector mechanisms. J Immunol *162*, 345-351.

Jenkins, S.J., Hewitson, J.P., Jenkins, G.R., and Mountford, A.P. (2005). Modulation of the host's immune response by schistosome larvae. Parasite Immunol *27*, 385-393.

Ji, J., Masterson, J., Sun, J., and Soong, L. (2005). CD4+CD25+ regulatory T cells restrain pathogenic responses during Leishmania amazonensis infection. J Immunol *174*, 7147-7153.

Karanja, D.M., Hightower, A.W., Colley, D.G., Mwinzi, P.N., Galil, K., Andove, J., and Secor, W.E. (2002). Resistance to reinfection with Schistosoma mansoni in occupationally exposed adults and effect of HIV-1 co-infection on susceptibility to schistosomiasis: a longitudinal study. Lancet *360*, 592-596.

Kelly, E.A., and Colley, D.G. (1988). In vivo effects of monoclonal anti-L3T4 antibody on immune responsiveness of mice infected with Schistosoma mansoni. Reduction of irradiated cercariae-induced resistance. J Immunol *140*, 2737-2745.

King, C.H. (2006). Schistosomiasis. In Tropical Infectious Diseases (Philadelphia, ELSEVIER), pp. 1341-1348.

Lamb, E.W., Crow, E.T., Lim, K.C., Liang, Y.S., Lewis, F.A., and Davies, S.J. (2007). Conservation of CD4+ T cell-dependent developmental mechanisms in the blood fluke pathogens of humans. Int J Parasitol *37*, 405-415.

Maggi, E., Cosmi, L., Liotta, F., Romagnani, P., Romagnani, S., and Annunziato, F. (2005). Thymic regulatory T cells. Autoimmun Rev *4*, 579-586.

McKee, A.S., and Pearce, E.J. (2004). CD25+CD4+ cells contribute to Th2 polarization during helminth infection by suppressing Th1 response development. J Immunol *173*, 1224-1231.

Mills, K.H., and McGuirk, P. (2004). Antigen-specific regulatory T cells--their induction and role in infection. Semin Immunol *16*, 107-117.

Miyara, M., and Sakaguchi, S. (2007). Natural regulatory T cells: mechanisms of suppression. Trends Mol Med *13*, 108-116.

Montesano, M.A., Colley, D.G., Willard, M.T., Freeman, G.L., Jr., and Secor, W.E. (2002). Idiotypes expressed early in experimental Schistosoma mansoni infections predict clinical outcomes of chronic disease. J Exp Med *195*, 1223-1228.

Mountford, A.P., and Trottein, F. (2004). Schistosomes in the skin: a balance between immune priming and regulation. Trends Parasitol *20*, 221-226.

Nylen, S., Maurya, R., Eidsmo, L., Manandhar, K.D., Sundar, S., and Sacks, D. (2007). Splenic accumulation of IL-10 mRNA in T cells distinct from CD4+CD25+ (Foxp3) regulatory T cells in human visceral leishmaniasis. J Exp Med *204*, 805-817.

Organization, W.H. (1993). The Control of Schistosomiasis. In WHO Technical Report, W.E. Committee, ed. (Geneva, WHO).

Osman, A., Niles, E.G., and LoVerde, P.T. (2001). Identification and characterization of a Smad2 homologue from Schistosoma mansoni, a transforming growth factor-beta signal transducer. J Biol Chem *276*, 10072-10082.

Osman, A., Niles, E.G., Verjovski-Almeida, S., and LoVerde, P.T. (2006). Schistosoma mansoni TGF-beta receptor II: role in host ligand-induced regulation of a schistosome target gene. PLoS Pathog *2*, e54.

Parham, P. (2005). The Immune System, Second Edition edn (Garland Science).

Pearce, E.J. (2003). Progress towards a vaccine for schistosomiasis. Acta Tropica *86*, 309-313.

Pearce, E.J. (2005). Priming of the immune response by schistosome eggs. Parasite Immunol *27*, 265-270.

Pearce, E.J., Caspar, P., Grzych, J.M., Lewis, F.A., and Sher, A. (1991). Downregulation of Th1 cytokine production accompanies induction of Th2 responses by a parasitic helminth, Schistosoma mansoni. J Exp Med *173*, 159-166.

Pearce, E.J., and MacDonald, A.S. (2002). The immunobiology of schistosomiasis. Nat Rev Immunol *2*, 499-511.

Ramaswamy, K., Kumar, P., and He, Y.X. (2000). A role for parasite-induced PGE2 in IL-10-mediated host immunoregulation by skin stage schistosomula of Schistosoma mansoni. J Immunol *165*, 4567-4574.

Rao, K.V., and Ramaswamy, K. (2000). Cloning and expression of a gene encoding Sm16, an anti-inflammatory protein from Schistosoma mansoni. Mol Biochem Parasitol *108*, 101-108.

Rouse, B.T. (2007). Regulatory T cells in health and disease. J Intern Med *262*, 78-95.
Rubtsov, Y.P., and Rudensky, A.Y. (2007). TGFbeta signalling in control of T-cell-mediated self-reactivity. Nat Rev Immunol *7*, 443-453.

Sacks, D., and Noben-Trauth, N. (2002). The immunology of susceptibility and resistance to Leishmania major in mice. Nat Rev Immunol *2*, 845-858.

Sher, A., Coffman, R.L., Hieny, S., and Cheever, A.W. (1990a). Ablation of eosinophil and IgE responses with anti-IL-5 or anti-IL-4 antibodies fails to affect immunity against Schistosoma mansoni in the mouse. J Immunol *145*, 3911-3916.

Sher, A., Coffman, R.L., Hieny, S., Scott, P., and Cheever, A.W. (1990b). Interleukin 5 is required for the blood and tissue eosinophilia but not granuloma formation induced by infection with Schistosoma mansoni. Proc Natl Acad Sci U S A *87*, 61-65.

Shokeir, A.A., and Hussein, M.I. (1999). The urology of Pharaonic Egypt. BJU Int *84*, 755-761.

Smythies, L.E., Coulson, P.S., and Wilson, R.A. (1992). Monoclonal antibody to IFN-gamma modifies pulmonary inflammatory responses and abrogates immunity to Schistosoma mansoni in mice vaccinated with attenuated cercariae. J Immunol *149*, 3654-3658.

Snapper, C.M.a.F., F.D. (1999). Immunoglobulin Class Switching, Vol Chapter 24, Fourth Edition edn (Philadelphia, Lippincott-Raven Publishers).

Sojka, D.K., Huang, Y.H., and Fowell, D.J. (2008). Mechanisms of regulatory T-cell suppression - a diverse arsenal for a moving target. Immunology.

Stenger, R.J., Warren, K.S., and Johnson, E.A. (1967). An electron microscopic study of the liver parenchyma and schistosome pigment in murine hepatosplenic schistosomiasis mansoni. Am J Trop Med Hyg *16*, 473-482.

Uhlig, H.H., Coombes, J., Mottet, C., Izcue, A., Thompson, C., Fanger, A., Tannapfel, A., Fontenot, J.D., Ramsdell, F., and Powrie, F. (2006). Characterization of Foxp3+CD4+CD25+ and IL-10-secreting CD4+CD25+ T cells during cure of colitis. J Immunol *177*, 5852-5860.

Vella, A.T., and Pearce, E.J. (1992). CD4+ Th2 response induced by Schistosoma mansoni eggs develops rapidly, through an early, transient, Th0-like stage. J Immunol *148*, 2283-2290.

Vignali, D.A., Crocker, P., Bickle, Q.D., Cobbold, S., Waldmann, H., and Taylor, M.G. (1989). A role for CD4+ but not CD8+ T cells in immunity to Schistosoma mansoni induced by 20 krad-irradiated and Ro 11-3128-terminated infections. Immunology *67*, 466-472.

Weiner, H.L. (2001a). Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. Immunol Rev *182*, 207-214.

Weiner, H.L. (2001b). Oral tolerance: immune mechanisms and the generation of Th3-type TGF-beta-secreting regulatory cells. Microbes Infect *3*, 947-954.

Wilson, M.S., Mentink-Kane, M.M., Pesce, J.T., Ramalingam, T.R., Thompson, R., and Wynn, T.A. (2007). Immunopathology of schistosomiasis. Immunol Cell Biol *85*, 148-154.

Wilson, R.A., Coulson, P.S., Betts, C., Dowling, M.A., and Smythies, L.E. (1996). Impaired immunity and altered pulmonary responses in mice with a disrupted interferon-gamma receptor gene exposed to the irradiated Schistosoma mansoni vaccine. Immunology *87*, 275-282.

Wynn, T.A. (2003). IL-13 effector functions. Annu Rev Immunol *21*, 425-456.

Wynn, T.A. (2004). Fibrotic disease and the T(H)1/T(H)2 paradigm. Nat Rev Immunol *4*, 583-594.

Wynn, T.A., Cheever, A.W., Williams, M.E., Hieny, S., Caspar, P., Kuhn, R., Muller, W., and Sher, A. (1998). IL-10 regulates liver pathology in acute murine Schistosomiasis mansoni but is not required for immune down-modulation of chronic disease. J Immunol *160*, 4473-4480.

Wynn, T.A., Oswald, I.P., Eltoum, I.A., Caspar, P., Lowenstein, C.J., Lewis, F.A., James, S.L., and Sher, A. (1994). Elevated expression of Th1 cytokines and nitric oxide synthase in the lungs of vaccinated mice after challenge infection with Schistosoma mansoni. J Immunol *153*, 5200-5209.

Wynn, T.A., Thompson, R.W., Cheever, A.W., and Mentink-Kane, M.M. (2004). Immunopathogenesis of schistosomiasis. Immunol Rev *201*, 156-167.

Yang, Z., Day, Y.J., Toufektsian, M.C., Xu, Y., Ramos, S.I., Marshall, M.A., French, B.A., and Linden, J. (2006). Myocardial infarct-sparing effect of adenosine A2A receptor activation is due to its action on CD4+ T lymphocytes. Circulation *114*, 2056-2064.